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Petition for the Determination of Nonregulated Status for Insect Resistant and Herbicide-Tolerant DP23211 Maize

Volume 1 - Petition

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OECD Unique Identifier: DP-Ø23211-2

Updated Submission date: July 20, 2020

Does Not Contain Confidential Business Information

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article, DP-Ø23211-2 maize (DP23211) not be regulated under 7 CFR §340.

Sally A. Catron — 7/20/2020

Sally A. Catron — Date

Release of Information

Pioneer Hi-Bred International, Inc. ("Pioneer", a member of the Corteva Agriscience Group of Companies), is submitting the information in this assessment for review by the USDA as part of the regulatory process. By submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C. section 522 or USDA's implementation regulations (7 CFR §340). In the event that USDA receives a FOIA request covering all or some of the information in this submission, Pioneer expects that, in advance of the release of the document(s), USDA will provide Pioneer with a copy of the material proposed to be released and the opportunity to object to the release of any information based upon appropriate legal grounds, e.g., responsiveness, confidentiality, or otherwise. Pioneer expects that no information that has been identified as confidential business information (CBI) will be provided to any third party. Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without prior notice and consent from Pioneer.

Certification

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article, DP-Ø23211-2 maize (DP23211) not be regulated under 7 CFR §340.

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners, which are favorable and unfavorable to the petition.

Sally A. Catron 7/25/2022

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Abbreviations, Acronyms, and Definitions

AACC	American Association of Cereal Chemists
ADF	Acid detergent fiber
AOAC	AOAC International
AOCS	American Oil Chemists' Society
at-T9	Terminator region from an *Arabidopsis thaliana* putative gene of the mannose-binding protein superfamily
BAPNA	Benzoyl-DL-arginine-p-nitroanilide-hydrochloride
BBMV	Brush border membrane vesicles
BC1F1	Backcross 1, first filial cross
BC2F2	Backcross 2, first filial cross
bp	Base pair
BPPD	Biopesticides and Pollution Prevention Division
*Bst*E II	*Bst*E II restriction enzyme from *Bacillus stearothermophilus* ET
BSV(AY)	Promoter region from the banana streak virus (acuminata Yunnan strain) genome
Bt	*Bacillus thuringiensis*
CaMV35S Terminator	35S terminator region from cauliflower mosaic virus
CBI	Confidential Business Information
CDM	Codling moth
CEW	Corn earworm
CFB	Crucifer flea beetle
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
CNV	Convergent lady beetle
CMAC	Pink spotted lady beetle
colE1 *ori*	Origin of replication region from *Escherichia coli*
CPB	Colorado potato beetle
COMPARE	Comprehensive Protein Allergen Resource
Cos	Cohesive ends from lambda bacteriophage DNA
CRW	Corn rootworm
Cry	Crystalline
ctl	Central control operon region from bacteria
DI	Deionized
DNA	Deoxyribonucleic acid
DP23211	Maize event DP-Ø23211-2
DsRed2	Modified red fluorescent protein gene from *Discosoma sp.* (coral anemone) with an internal *Bst*E II restriction site removed

Abbreviations, Acronyms, and Definitions (continued)

DsRed2	Modified red fluorescent protein from *Discosoma sp.*
dsRNA	Double-stranded ribonucleic acid
dUTP	Deoxyuridine-triphosphatase
dvssj1	Smooth septate junction protein 1 gene from *Diabrotica virgifera virgifera*
DvSSJ1	Smooth septate junction protein 1 from *Diabrotica virgifera virgifera*
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
ECB	European corn borer
EEC	Estimated environmental concentration
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
EPL BAS	EPL Bio Analytical Services
ETS	Excellence Through Stewardship®
F1	First filial generation (hybrid)
FAME	Fatty acid methyl ester
FAW	Fall armyworm
FDA	Food and Drug Administration of the United States
FDR	False Discovery Rate
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FLP	Flippase protein
FOIA	Freedom of Information Act
FR	Federal Register
FRT1	Flippase recombination target site from *Saccharomyces cerevisiae*
FRT87	Modified flippase recombination target site derived from *Saccharomyces cerevisiae*
%FW	Percent fresh weight
GC/FID	Gas chromatography flame ionization detection
GE	Genetically engineered
GLP	Good Laboratory Practices
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ICP-OES	Inductively coupled plasma optical emission spectroscopy
In2-1 Terminator	Terminator region from the *Zea mays In2-1* gene
In2-2 Promoter	Promoter region from the *Zea mays In2-2* gene
ipd072Aa	Insecticidal protein gene from *Pseudomonas chlororaphis*
IPD072Aa	Insecticidal protein from *Pseudomonas chlororaphis*

Abbreviations, Acronyms, and Definitions (continued)

IPM	Integrated pest management
kb	Kilobase
kDa	Kilodalton
Kpn I	Restriction site/enzyme from *Klebsiella pneumoniae* OK8
LB	Left border
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LLOQ	Lower limit of quantification
*lox*P	Bacteriophage P1 recombination site recognized by Cre recombinase
LTP	Lipid transfer protein
Ltp2	Promoter region from the *Hordeum vulgare* (barley) aleurone-specific lipid transfer protein gene
M	Molar
MBB	Mexican bean beetle
MBD	Mealy bug destroyer
mM	Millimolar
µg	Microgram
MOA	Mode of action
MOE	Margin of exposure
mo-Flp Exon 1	Maize-optimized exon 1 of the flippase gene from *Saccharomyces cerevisiae*
mo-Flp Exon 2	Maize-optimized exon 2 of the flippase gene from *Saccharomyces cerevisiae*
mo-pat	Maize optimized phosphinothricin acetyl transferase gene from *Streptomyces viridochromogenes*
MS/MS	Tandem mass spectrometry
MWM	Mealworm
NCR	Northern corn rootworm
NDF	Neutral detergent fiber
NIL	Near isoline
NGS	Next Generation Sequencing
nm	Nanometer
NOEC	No-observed effect concentration
NOED	No-observed effect dose
NOEDD	No-observed effect dietary dose
non-GE	Non-genetically engineered

Abbreviations, Acronyms, and Definitions (continued)

nos Promoter	Promoter region from the *Agrobacterium tumefaciens* Ti plasmid nopaline synthase gene
nt	Nucleotide
NTO	Non-target organism
ODP2	Ovule development protein 2
OECD	Organisation for Economic Cooperation and Development
ORF	Open reading frame
*ori*T	Origin of transfer region from bacteria
*ori*V	Origin of replication region from bacteria
os-actin Intron	Intron region from the *Oriza Sativa* (rice) actin gene
os-actin Promoter	Promoter region from the *Oriza Sativa* (rice) actin gene
pat	Phosphinothricin acetyl transferase gene
PAT	Phosphinothricin acetyl transferase protein
PBN	Premarket Biotechnology Notice
PCR	Polymerase chain reaction
PH1V5T	Pioneer proprietary inbred line
PH2SRH	Pioneer proprietary inbred line
PHR03	Pioneer proprietary inbred line
*pin*II Terminator	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene
PIP	Plant-Incorporated Protectant
PL	Painted lady butterfly
pmi	Phosphomannose isomerase gene from *Escherichia coli* including 5' and 3' untranslated regions
PMI	Phosphomannose isomerase protein
P-value	Probability value
R1	Reproductive stage 1
R2	Reproductive stage 2
R3	Reproductive stage 3
R4	Reproductive stage 4
R5	Reproductive stage 5
R6	Reproductive stage 6
RB	Right border
RFB	Red flour beetle
RNA	Ribonucleic acid
RNAi	RNA interference

Abbreviations, Acronyms, and Definitions (continued)

RVB	Rove beetle
SbS	Southern-by-Sequencing
sb-gkaf Terminator	Terminator region from the *Sorghum bicolor* (sorghum) γ-kafarin gene
sb-ubi Terminator	Terminator region from the *Sorghum bicolor* (sorghum) ubiquitin gene
SCR	Southern corn rootworm
SFB	Striped flea beetle
siRNA	Small interfering RNA
SoA	Site of action
SNP	Single nucleotide polymorphism
spc	Spectinomycin resistance gene from bacteria
SSI	Site-specific integration
SSJ	Smooth septate junction
st-LS1 Intron	Intron region from the *Solanum tuberosum* (potato) *LS1* gene
SWM	Superworm
T0	Transformation generation
T1	First selfed generation
T2	Second selfed generation
T3	Third selfed generation
T4	Fourth selfed generation
T5	Fifth selfed generation
TBHQ	Tert-butylhydroquinone
T-DNA	Transfer DNA
TDF	Total dietary fiber
tetA	Tetracycline resistance gene from bacteria
tetR	Tetracycline resistance regulation gene from bacteria
trfA	Trans-acting replication gene from bacteria
*ubi*ZM1 5' UTR	Untranslated region from the *Zea Mays* ubiquitin gene 1
*ubi*ZM1 Intron	Intron region from the *Zea Mays* ubiquitin gene 1
*ubi*ZM1 Promoter	Promoter region from the *Zea Mays* ubiquitin gene 1
UPLC	Ultra performance liquid chromatography
USDA-APHIS	United States Department of Agriculture - Animal and Plant Health Inspection Service
UTR	Untranslated region
UV	Ultraviolet

Abbreviations, Acronyms, and Definitions (continued)

V2	Vegetative stage 2
V3	Vegetative stage 3
V4	Vegetative stage 4
V5	Vegetative stage 5
V6	Vegetative stage 6
V7	Vegetative stage 7
V8	Vegetative stage 8
V9	Vegetative stage 9
VT	Vegetative tassel stage
*vir*C1	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
*vir*D1	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
WCR	Western corn rootworm
WUS	Protein encoded by the maize *Wuschel2* gene
zm-odp2	Ovule development protein 2 gene from *Zea mays*
zm-wus2	*Wuschel2* gene from *Zea mays*

Executive Summary

Pioneer Hi-Bred International, Inc. (Pioneer, a member of Corteva Agriscience Group of Companies) is submitting a Petition for Determination of Nonregulated Status for insect resistant and herbicide tolerant maize event DP-Ø23211-2, hereafter referred to as DP23211 maize. Pioneer requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that DP23211 maize, DP23211 maize progeny, and any crosses of DP23211 maize with other nonregulated maize no longer be considered regulated articles under 7 CFR §340.

DP23211 maize was genetically engineered to produce DvSSJ1 double-stranded ribonucleic acid (dsRNA) and the IPD072Aa protein for control of corn rootworm (CRW) pests, the phosphinothricin acetyltransferase (PAT) protein for tolerance to glufosinate-ammonium herbicides, and the phosphomannose isomerase (PMI) protein used as a selectable marker.

The DvSSJ1 dsRNA produced in DP23211 maize is targeted to match a portion of the smooth septate junction protein 1 (*dvssj1*) gene from western corn rootworm (WCR, *Diabrotica virgifera virgifera*) to down-regulate expression of the DvSSJ1 protein in the mid-gut of WCR via RNA interference (RNAi). DP23211 maize produces DvSSJ1 dsRNA which, when ingested by WCR, results in suppression of the DvSSJ1 protein in the intestinal lining. Reduction in DvSSJ1 protein expression and subsequent loss of formation of the gut epithelium barrier and cellular deformities are lethal to WCR (Hu et al., 2019).

The IPD072Aa protein, encoded by the *ipd072Aa* gene, confers control of CRW pests when expressed in plants by causing disruption of the midgut epithelium. The *ipd072Aa* gene was identified and cloned from a *Pseudomonas chlororaphis* strain that was cultured from a soil sample (Schellenberger et al., 2016).

DP23211 maize also contains the *mo-pat* gene which encodes the phosphinothricin acetyltransferase (PAT) protein. The PAT protein confers tolerance to the herbicidal active ingredient glufosinate-ammonium at current labeled rates. The PAT protein present in DP23211 maize is identical to the corresponding protein found in previously authorized events across several different crops that are currently in commercial use.

The *pmi* gene in DP23211 maize expresses the PMI protein as a selectable marker and is identical to the corresponding protein found in previously authorized events across several crops that are currently in commercial use (Negrotto et al., 2000). The mode of action and safety of the PAT and PMI proteins have already been assessed (CERA, 2011a; Hérouet *et al.*, 2005; OECD, 1999; Reed *et al.*, 2001).

DP23211 maize was generated using *Agrobacterium*-mediated transformation with plasmid PHP74643 containing the DvSSJ1 fragment cassette and *ipd072Aa*, *mo-pat,* and *pmi* gene

cassettes. Molecular characterization of DP23211 maize by Southern blot analysis and a Next Generation Sequencing (NGS) method known as Southern-by-Sequencing (SbS™ technology, hereafter referred to as SbS) confirmed that the inserted DNA in DP23211 maize is stable and equivalent across multiple generations during the breeding process. Segregation analysis of DP23211 maize confirmed the Mendelian inheritance of the DvSSJ1 fragment cassette and *ipd072Aa*, *mo-pat,* and *pmi* gene cassettes. Bioinformatics analysis of open reading frames (ORFs) identified no homology to known allergens or toxins for the putative translated ORFs at the DP23211 maize insertion site.

The potential for allergenicity and toxicity of DP23211 maize was evaluated by examining the allergenic potential of maize as a crop and by assessing the allergenic and toxic potential of the DvSSJ1 dsRNA, and the IPD072Aa, PAT and PMI proteins. Maize is not a common allergenic food and the modification of DP23211 maize is not expected to alter the allergenic potential of maize. Nucleic acids, including dsRNA, are normal components of human and animal diets, and have a history of safe consumption in food and feed. The molecular target of DvSSJ1 dsRNA is specific to *Diabrotica* species within Chrysomelidae, comparisons of DvSSJ1 dsRNA 21-nucleotide (nt) small interfering RNAs (siRNAs) to mammalian, avian and fish transcriptomes did not identify 21-nt siRNA exact matches. Physical, enzymatic, biochemical and molecular barriers to exposure of ingested small RNAs is likely to further reduce potential exposure to DvSSJ1 dsRNA consumed in food and feed from DP23211 maize.

The IPD072Aa protein expressed in DP23211 maize was derived from a common soil bacterium. The IPD072Aa protein did not meet the assessment criteria for known human and animal toxins or allergens. Expression levels of IPD072Aa were measured in edible maize tissues and exposure calculations were performed for humans and livestock.

The PAT and PMI proteins in DP23211 maize are identical to the PAT and PMI proteins found in previously authorized events that are currently in commercial use (USDA-APHIS, 2001; USDA-APHIS, 2005; USDA-APHIS, 2013; Vlachos and Huber, 2011; Ward and Huber, 2007). The PAT and PMI proteins have been extensively reviewed in numerous preceding regulatory submissions and no evidence of acute toxicity, glycosylation, or identity to known allergens or toxins have previously been identified. The level of expression of the PAT and PMI proteins in DP23211 maize does not significantly raise exposure of humans or animals when compared against exposure through consumption of previously authorized genetically engineered (GE) maize containing the PAT or PMI protein.

Nutrient composition analyses of grain and forage (79 total analytes) were conducted to compare the composition of DP23211 maize to that of a control maize line (defined as non-GE, near-isoline in this petition) and 14 conventional maize varieties (defined as non-GE hybrids used in commercial production in this petition). These analyses were used to evaluate any changes in

the levels of key nutrients, anti-nutrients and secondary metabolites. Based on the results of the compositional evaluation, the grain and forage of DP23211 maize is comparable to conventional maize. Use of DP23211 maize is not expected to result in any significant impact on raw or processed maize commodities.

Agronomic performance assessments for DP23211 maize were conducted in replicated field studies at a total of 12 locations in the United States and Canada. The following characteristics were measured: early stand count, days to flowering, pollen viability, days to maturity, lodging, final stand count, dropped ears, yield, harvest grain moisture, and 100-kernel weight. Additionally, biotic and abiotic observations were taken by evaluating insect damage incidence, plant pathogen incidence, and abiotic stress at each site during the growing season. Seed germination and viability data were also collected in laboratory experiments. Analysis of agronomic data showed no statistically significant differences (False Discovery Rate (FDR) adjusted P-value > 0.05) between DP23211 maize and non-GE, near isoline control maize lines, except for three characteristics. Days to flowering and final population mean values were significantly different from the non-GE near isoline control. The mean values for days to flowering and final population are within the reference range for each measurement which indicates that these differences are likely not biologically meaningful.

Overall analysis showed the agronomic comparability of DP23211 maize to conventional maize. In addition, DP23211 maize has been field tested over five years in the United States and Puerto Rico. All releases in the United States have occurred under field permits and notifications granted by USDA - APHIS. All field trials of DP23211 maize were observed for naturally occurring insects or diseases, and no unexpected differences between DP23211 maize and control maize were observed. Together, these data support the conclusion that DP23211 maize is unlikely to pose a greater plant pest risk than conventional maize.

The potential environmental impact of the introduction of DP23211 maize considered the potential for DP23211 maize to become weedy or invasive; and the potential for gene flow to sexually compatible wild relatives. In general, maize does not possess weediness characteristics and is not considered a weedy or invasive species. Analyses indicated that DP23211 maize was comparable to conventional maize with respect to agronomic characteristics measured. Therefore, DP23211 maize does not exhibit characteristics that would indicate it is any more likely than conventional maize to become a weed or plant pest. The potential for gene flow examined maize pollination biology and the hybridization potential and geographic overlap of maize wild relatives. While maize does possess some pollination characteristics favorable to gene flow, the distribution of wild relative populations is limited in the United States. Therefore, it is unlikely that the inserted DNA in DP23211 maize would be introgressed into wild relative populations.

An analysis was performed and is focused on the insecticidal DvSSJ1 dsRNA and the IPD072Aa protein expressed by DP23211 maize and assessed the potential effect to non-target organisms (NTOs). Estimated environmental concentrations (EECs) of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize were determined using worst-case assumptions (e.g., maximum concentrations of DvSSJ1 dsRNA and the IPD072Aa protein in relevant DP23211 maize tissues) to determine potential exposure for NTOs, including pollinators and pollen feeders, soil-dwelling organisms, aquatic organisms, predators and parasitoids, and insectivorous birds. Several factors that reduce potential exposure to NTOs under more realistic environmental conditions were considered and used to refine EECs when needed to understand environmentally relevant concentrations. Environmental risk was characterized by comparing Tier I hazard study results to worst-case EECs or refined EECs to calculate the margins of exposure (MOEs) for the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize. Based on the MOE values for DP23211 maize, the DvSSJ1 dsRNA and the IPD072Aa protein are not expected to be harmful to beneficial NTO populations at environmentally relevant concentrations.

The data and information contained herein supports the conclusion that DP23211 maize does not present a plant pest risk and is not otherwise deleterious to the environment. Therefore, Pioneer requests that APHIS grant the request for a determination of nonregulated status for DP23211 maize, DP23211 maize progeny, and any crosses of DP23211 maize with other nonregulated maize.

I. Rationale for the Development of DP23211 Maize

I-A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR §340.6

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772), to prevent the introduction or dissemination of plant pests into or within the United States. 7 CFR §340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations in 7 CFR §340.6 provide that an applicant may petition APHIS to evaluate submitted data on the GE crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting data for GE CRW-resistant and glufosinate-tolerant DP-Ø23211-2 (DP23211) maize and requests a determination from USDA-APHIS that DP23211 maize, DP23211 maize progeny, and any crosses with other nonregulated maize no longer be considered regulated articles under 7 CFR §340.

I-B. Rationale for the Development of DP23211 Maize

DP23211 maize was genetically engineered to produce DvSSJ1 double-stranded Ribonucleic Acid (dsRNA) and the IPD072Aa protein for control of CRW, the phosphinothricin acetyltransferase (PAT) protein for tolerance to glufosinate-ammonium herbicides, and the phosphomannose isomerase (PMI) protein used as a selectable marker.

Maize is the largest crop grown in the United States in terms of both acreage and net value, with multiple downstream uses for feed, fuel, and food. In 2018, maize was planted on approximately 89.1 million acres (USDA-NASS, 2018a). The United States is one of the world's largest producers and a leading exporter of maize, underscoring the crop's importance both nationally and globally (USDA-ERS, 2018a).

DP23211 maize produced DvSSJ1 dsRNA and the insect-active protein IPD072Aa have been demonstrated to be efficacious against CRW pests, including western corn rootworm (*Diabrotica virgifera virgifera)* (WCR). WCR is among the most serious pests of maize in the United States with economic losses of greater than $1 billion annually from both management costs and yield loss (Metcalf, 1986; PHI, 2010; Shrestha et al., 2018). WCR damage has historically been managed with crop rotation, broad-spectrum soil insecticides, and transgenic crops expressing crystalline (Cry) proteins from *Bacillus thuringiensis* (*Bt*). As adoption of *Bt* maize has increased, the selection on target insects to develop resistance has become greater (Cullen et al., 2013). Insect resistance to transgenic traits can pose a threat to the long-term durability of *Bt* crops.

The PAT protein confers tolerance to glufosinate ammonium, the active ingredient in phosphinothricin herbicides (CERA - ILSI Research Foundation, 2016). The PMI protein was used as a selectable marker during DP23211 maize development

As reduced performance of Cry3 and Cry34/35Ab1 proteins in maize has been reported in the scientific literature (Gassmann et al., 2016; Jakka et al., 2016), new modes of action (MOA) are important for maintaining sustainable and durable CRW management (Gassmann et al., 2016; Niu et al., 2017). DP23211 maize diversifies the currently available *Bt* protein-based MOA for CRW control through the combination of an RNA-mediated MOA with an alternative protein MOA. DP23211 maize provides farmers with an additional control option for CRW pests to protect maize grain yield that is important to United States' farmers and the United States' economy.

I-C. Prior Environmental Release and Submissions to Other Regulatory Agencies

DP23211 maize has been field tested in the United States and Puerto Rico over 5 years in more than 150 separate plantings as authorized by the USDA-APHIS permits and notifications (Appendix 1).

A voluntary safety and nutritional assessment of DP23211 maize was submitted to the FDA's Center for Food Safety and Applied Nutrition (FDA CFSAN) in 2019. Pioneer has also submitted a registration application to the EPA Biopesticides and Pollution Prevention Division (BPPD) under FIFRA Section 3. This submission included a petition for exemption from the requirement of a tolerance under the Federal Food, Drug, and Cosmetic Act (FFDCA) for the IPD072Aa protein. The DvSSJ1 dsRNA is exempt from the requirement of a tolerance under 40 CFR 174.507.

Corteva is committed to robust product stewardship prior to launch and continuing through product discontinuation. Corteva is a member of Excellence Through Stewardship® (ETS). Pioneer products are commercialized in accordance with Corteva policies regarding stewardship of those products and with ETS Product Launch Policy Stewardship Guidance

I-D. Maize Crop Cultivation in the United States and Usage

Maize is the largest crop grown in the United States in terms of acreage and net value. Maize has multiple downstream uses for feed, fuel, and food that are significant for U.S. and global supply. In 2018, 14.4 billion bushels of maize were produced in the United States from approximately 89.1 million planted acres, valued at $51.9 billion (USDA-NASS, 2018a; USDA-NASS, 2018b). This represents approximately 33% of the world's maize supply in 2018-2019 (USDA-FAS, 2019).

The U.S. is a major global exporter of maize at approximately 38% of the total trade market (USDA-FAS, 2019). The largest maize United States export markets in 2018-2019 were Mexico,

Japan, Colombia, South Korea, and Peru (NCGA, 2018a; U.S. Grains Council, 2019). United States exports accounted for 15.4% of the maize produced in 2018 and those exports were shipped to more than 70 countries (NCGA, 2018b; U.S. Grains Council, 2019).

A significant portion of maize cultivated in the United States is GE. In 2018, 92% of maize grown in the United States was planted to GE hybrids. Insect resistant hybrids accounted for approximately 82% of all maize acreage, which includes the percentage of insect resistant traits as well as stacked varieties (USDA-ERS, 2018b).

I-D.1. Maize Processing for Feed, Fuel, and Food Uses

Maize grain requires processing into its four major components: starch, germ, fiber, and protein (CRA, 2017) for downstream uses. Wet and dry milling processes are used to separate grain into components for food, feed, and fuel processing (OECD, 2002).

Wet milling starts with softening the kernel in hot water and sulfur dioxide prior to further fractionation and processing. Products from the wet milling process include germ meal, oil (further processed into margarine, cooking oil, baking and frying fats), corn gluten feed, corn gluten meal, and starch (further processed into ethanol and sweeteners) (OECD, 2002).

There are several means of dry milling maize grain, but by far the most widely used process begins with soaking the kernel in water to remove the pericarp and germ, followed by drying the remaining grain fraction before additional processing. Products from the dry milling process include flour, meal, germ meal, oil, beverage and fuel ethanol, distillers dried grain/solubles (DDG), flaking grits, hominy feed, and grits. Maize grain may also be cooked in alkali and finely ground to produce what is known as *masa*, which is used for tortillas and snack chips (OECD, 2002)

The production of fuel ethanol typically begins with dry milling of maize grain, cooking, saccharification, and fermentation to produce ethanol and the by-product DDG (OECD, 2002).

I-D.2. Feed Use of Maize

Approximately one third (33%) of the maize produced in the United States (5.3 billion bushels) is used for animal feed (NCGA, 2018b); (NCGA, 2019). A number of different products from the maize plant and from grain processing may be used as feed.

The whole maize plant or its residue from harvesting are frequently used as animal feed. Silage, derived from the above-ground portions of the maize plant, is an important feed ingredient for feedlot and dairy cattle and preserves more than 90% of nutrients. In 2017, 128 million tons of corn silage were produced on 6.43 million acres (Progressive Forage, 2018). In addition, stalks from harvested maize plants can be grazed by ruminants in the field (OECD, 2002).

Maize ears, without shelling (*i.e.*, removing the grain from the cob), can be ground directly for ruminant feed. When ears are shelled to remove the grain, remnant cobs can also be used in animal feed. Maize grain can be fed to animals with minimal processing and can be fed whole, rolled, ground, or steam flaked. Rolled or ground grain is fed to swine and poultry. Maize grain added to pet foods is ground, cooked, and pelleted or extruded (OECD, 2002).

Processed products from the milling and ethanol fermentation processes are also fed to livestock. A by-product of the wet milling process, corn gluten meal, is fed to ruminants, poultry, and swine (OECD, 2002). The ethanol fermentation process produces a co-product called (DDG) or corn gluten feed that is used as animal feed to dairy and beef cattle, poultry, and swine (USDA-ERS, 2009; USDA-ERS, 2010; USDA-NASS, 2007). Use of DDG in domestic livestock rations in 2017 was approximately 32 million metric tons (NCGA, 2018a).

I-D.3. Fuel Use of Maize

Maize is the primary feedstock used to produce ethanol in the United States; 25% of maize grain produced in 2018 was fermented into fuel ethanol (NCGA, 2018b). Data from the United States Energy Information Administration (EIA) estimates that in 2017 about 10% of the total volume of finished motor gasoline consumption (142.85 billion gallons) consumed in the United States contained fuel ethanol (US-EIA, 2018).

I-D.4. Food Use of Maize

Starch, oil, grits, bran, meal, and flour from maize wet and dry milling are primarily used in foods (OECD, 2002). A majority of starch is converted to sweeteners, such as corn syrup, high fructose corn syrup, maltodextrins, and dextrose, and also fermented into ethanol (OECD, 2002). In 2018, 459 million bushels (2.9% of total usage) of United States maize went to the production of high-fructose corn syrup as an end-use product (NCGA, 2018a). Maize produced in the United States was also used for production of starch, sweeteners, cereal/food, and beverage alcohol (NCGA, 2018a).

Starch is used for food such as bakery products/mixes, condiments, candies, and prepared (snack, dessert, meat) foods (CCUR, 2009). Sweeteners are used for soft drinks, candies, bakery products/mixes, condiments (jams, jellies, dressings), and prepared foods (CCUR, 2009). Whole maize is consumed as popcorn, sweet corn, and alkali processed grain for tortillas and snack chips (CCUR, 2009), though these uses comprise a very minor usage segment.

II. The Biology of Maize

II-A. Maize as a Crop

Biology documents on the non-Genetically Engineered (non-GE) (also referred to as "conventional") plant species, maize (*Zea Mays* L.), have been published by the Canadian Food Inspection Agency (CFIA, 1994) and by the Organization for Economic Co-operation and Development (OECD, 2003). These documents provide background on the biology of *Zea mays* including:

- information on use of maize as a crop plant
- taxonomic status of *Zea mays*
- identification methods
- reproductive biology
- centers of origin and diversity
- crosses, including intra- and inter-specific/genus crosses and gene flow
- agro-ecology, including information about cultivation, volunteers and weediness, soil ecology, and maize-insect interactions

Maize is extensively cultivated worldwide and has a long history of safe use. Maize grain and maize-derived products represent staple food and feed for a large portion of the global population (CFIA, 1994). No significant toxicity or allergenicity has been ascribed to any food or feed uses of maize, and maize has been described as a food that is likely to have low allergenicity (OECD, 2002). Maize is not included in the list of food allergy indications of the US Food and Drug Administration (US-FDA, 2006).

II-B. Description of the Non-Transformed Recipient Maize Line

A Pioneer proprietary line, PHR03 was used as the recipient line to produce DP23211 maize. Line PHR03 was chosen because it is receptive to transformation and is also an elite line (*i.e.*, Pioneer proprietary line used for commercial products).

III. Method of Development of DP23211 Maize

III-A. Description of Transformation, Selection, and Breeding Method

III-A.1. Transformation of DP23211 Maize

DP23211 maize was created by Site-Specific Integration (SSI) using two sequential transformation steps to (1) insert a specific integration site sequence (referred to as a "landing pad" sequence) at a specific location of the maize genome using Pioneer proprietary line PHR03 and (2) insert the intended expression cassettes from the plasmid PHP74643 T-DNA region into the landing pad in the maize genome.

The landing pad was created by microprojectile bombardment of maize tissue with plasmid PHP56614 which contains the flippase recombination target sites, FRT1 and FRT87, and the maize genome-derived sequences, *zm*-SEQ9 and *zm*-SEQ8. The I-CreI endonuclease expressed by plasmid PHP56614 created a double-stranded DNA break at a specified location in the genome, after which homologous recombination between the maize genome and *zm*-SEQ9 and *zm*-SEQ8 inserted the landing pad into the maize genome. Two additional plasmids, PHP21139 and PHP31729, were included in this transformation to improve plant regeneration, but were not incorporated into the maize genome. After molecular characterization, a line with the inserted landing pad and no unintended DNA insertions was selected and moved forward to the next step in the transformation process. Following *Agrobacterium*-mediated transformation with plasmid PHP74643, transfer of the intended expression cassettes from the plasmid PHP74643 T-DNA region into the SSI landing pad occurred via flippase-mediated recombination and exchange of the sequence between the FRT1 and FRT87 sites, resulting in the intended insertion containing the desired expression cassettes. Thus, the intended insertion in DP23211 maize comprises sequences from both PHP56614 and PHP74643 and includes the *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes and the DvSSJ1 fragment cassette.

III-A.1.1. Insertion of PHP56614 T-DNA Landing Pad

The first transformation step utilized microprojectile co-bombardment with three plasmids to insert the landing pad using an I-CreI-endonuclease-mediated gene-editing process. The I-CreI protein binds to a defined DNA target location based on specific protein-DNA interaction (Daboussi et al., 2015). Three plasmids PHP56614 (Figure 1), PHP21139 (Figure 3), and PHP31729 (Figure 4) were used to deliver the various components needed for the recombination processes and improved plant regeneration (Table 1).

Following biolistic transformation, the I-*Cre*I (PHP56614), *zm-wus2* (PHP21139), and *zm-odp2* (PHP31729) genes are transiently expressed without integration into the maize genome. The I-CreI endonuclease enzyme produced a double-stranded break at a targeted location in the maize genome between the endogenous *zm*-SEQ9 and *zm*-SEQ8 sequences. Following this, a native

cellular mechanism known as homology-directed repair (HDR) occurred via crossovers between the *zm*-SEQ9 and *zm*-SEQ8 sequences from the introduced PHP56614 and the identical *zm*-SEQ9 and *zm*-SEQ8 sequences naturally present in the maize genome. HDR thus introduced the *ubi*ZM1 promoter including the 5' untranslated region (5' UTR), FRT1, *npt*II gene, and FRT87 elements into the maize genome between the two maize sequences (*zm*-SEQ9 and *zm*-SEQ8). The expression of WUS2 (PHP21139) and ODP2 (PHP31729) allows for the improved regeneration of maize plants from the transformation process.

After transformation, regeneration of maize plants, and molecular characterization by SbS, a line with the landing pad and no unintended DNA sequence inserts was selected and advanced in the transformation process.

Table 1. Summary of Genetic Elements in the Landing Pad Transformation and Presence in DP23211 Maize

Plasmid	Genetic element	Description	Present in DP23211 Maize
PHP56614	*lox*P	Cre-recombination site	No
	I-*Cre*I	Maize-optimized I-*Cre*I endonuclease from *Chlamydomonas reinhardtii* modified to target a specific maize genome location	No
	*Lox*P	Cre recombination site	No
	zm-SEQ9	Genomic recognition site for HDR	Yes
	*ubi*ZM1	Promoter	Yes
	FRT1	Flippase recombination target site	Yes
	*npt*II	Neomycin phosphotransferase gene	No
	FRT87	Flippase recombination target site	Yes
	zm-SEQ8	Genomic recognition site for HDR	Yes
PHP21139	*zm-wus2*	Developmental gene for regeneration	No
PHP31729	*zm-odp2*	Developmental gene for regeneration	No

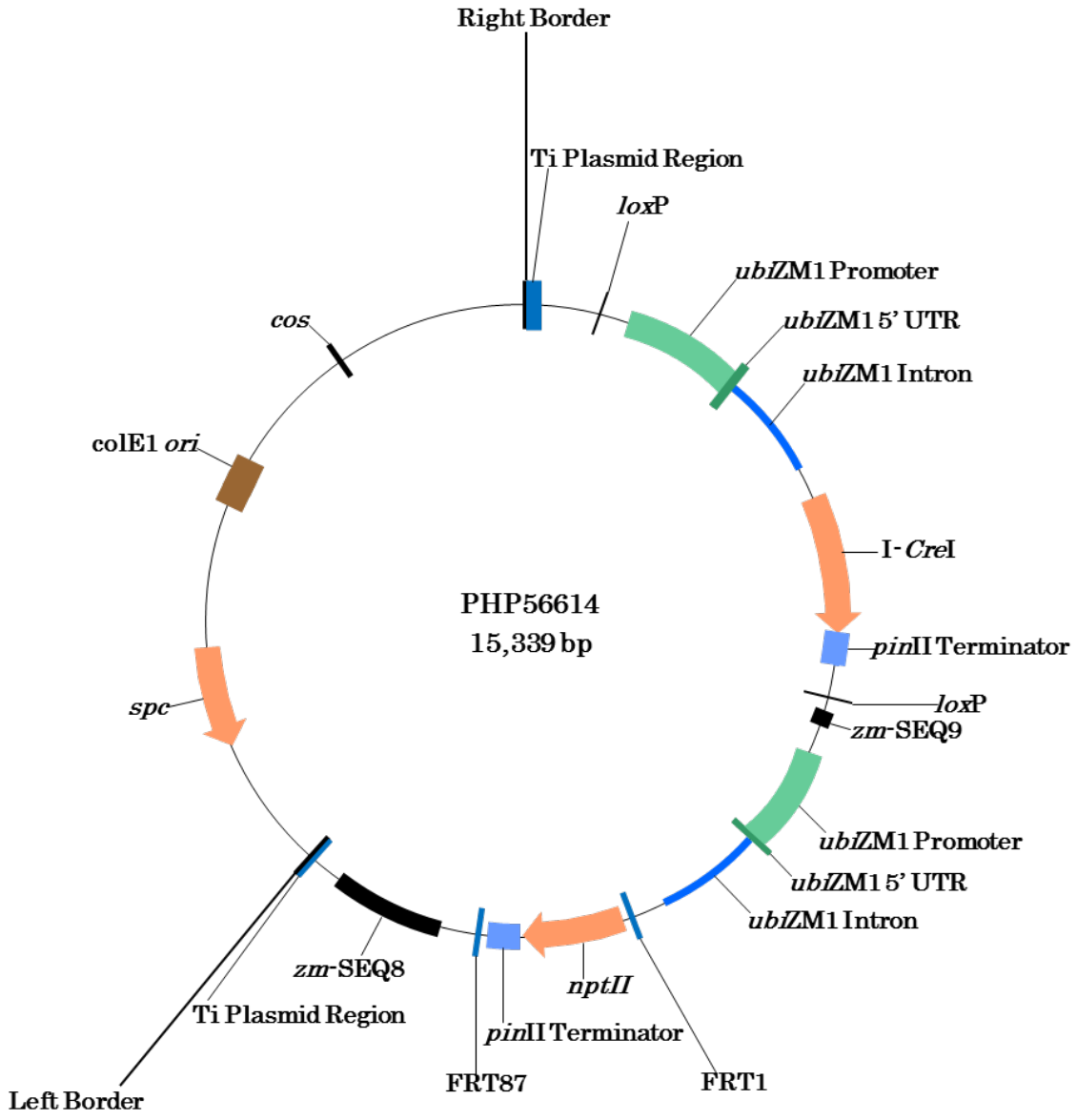

Figure 1. **Schematic Diagram of Plasmid PHP56614**
Schematic diagram of plasmid PHP56614 containing the I-*Cre*I and *nptII* gene cassettes, along with the *zm*-SEQ9, *zm*-SEQ8, FRT1, and FRT87 elements. The plasmid size is 15,339 bp. The region from *zm*-SEQ9 to *zm*-SEQ8 formed the SSI landing pad in the maize genome. The region between FRT1 and FRT87 was replaced by SSI with the intended DvSSJ1 fragment/gene-cassettes from the PHP74643 T-DNA (Figure 6) that is flanked by the same sites.

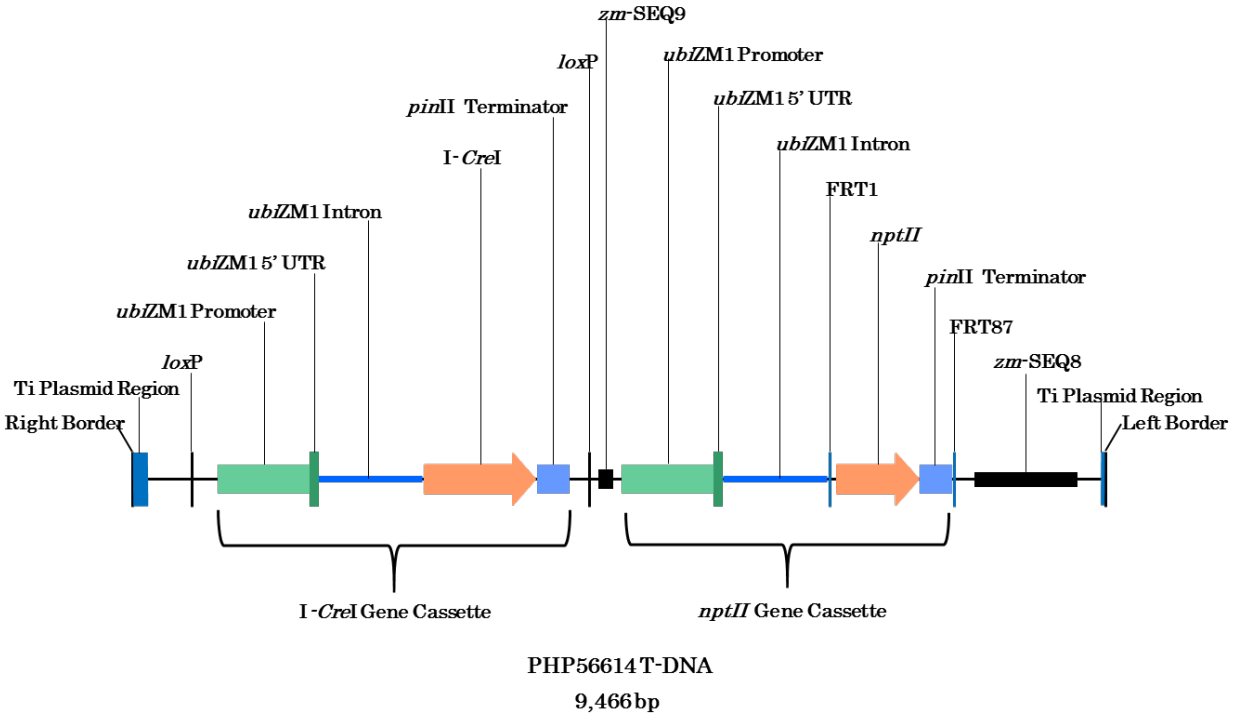

Figure 2. **Schematic Diagram of the T-DNA Region from Plasmid PHP56614**
Schematic diagram of the T-DNA region from plasmid PHP56614 containing the I-*Cre*I and *nptII* gene cassettes. The size of the T-DNA is 9,466 bp.

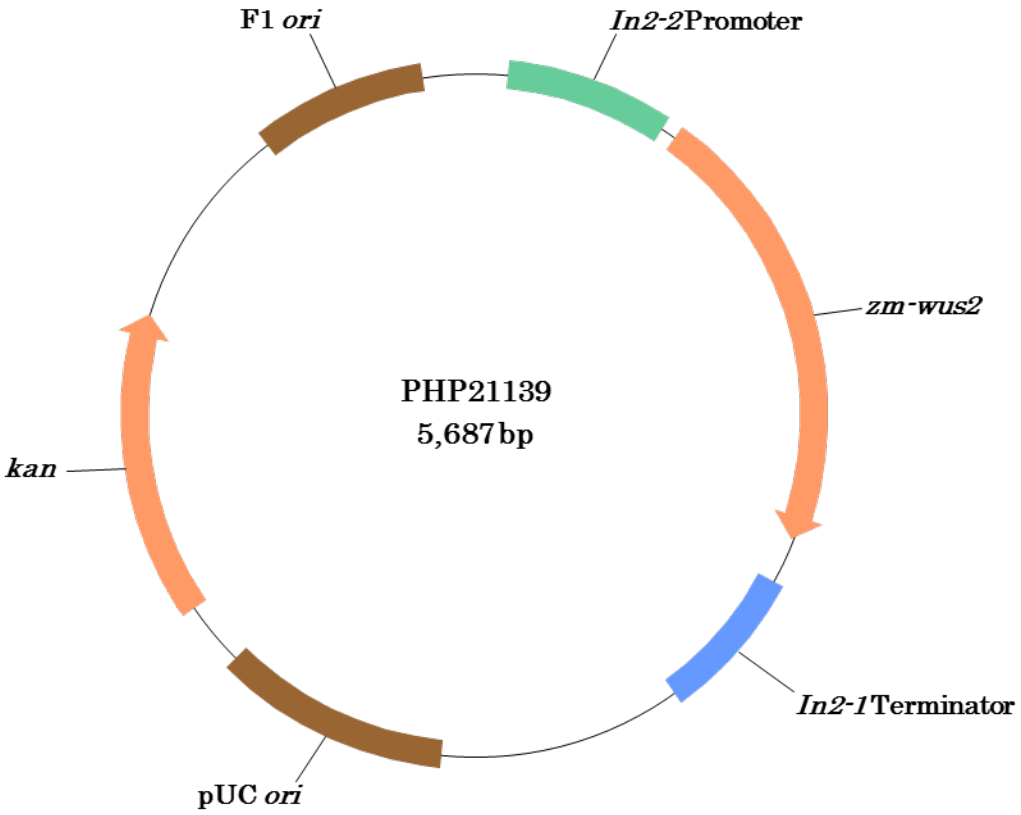

Figure 3. **Schematic Diagram of Plasmid PHP21139**
Schematic diagram of plasmid PHP21139 containing the *zm-wus2* gene cassette. The plasmid size is 5,687 bp. PHP21139 was used to enhance transformation and plant regeneration but was not incorporated into the maize genome.

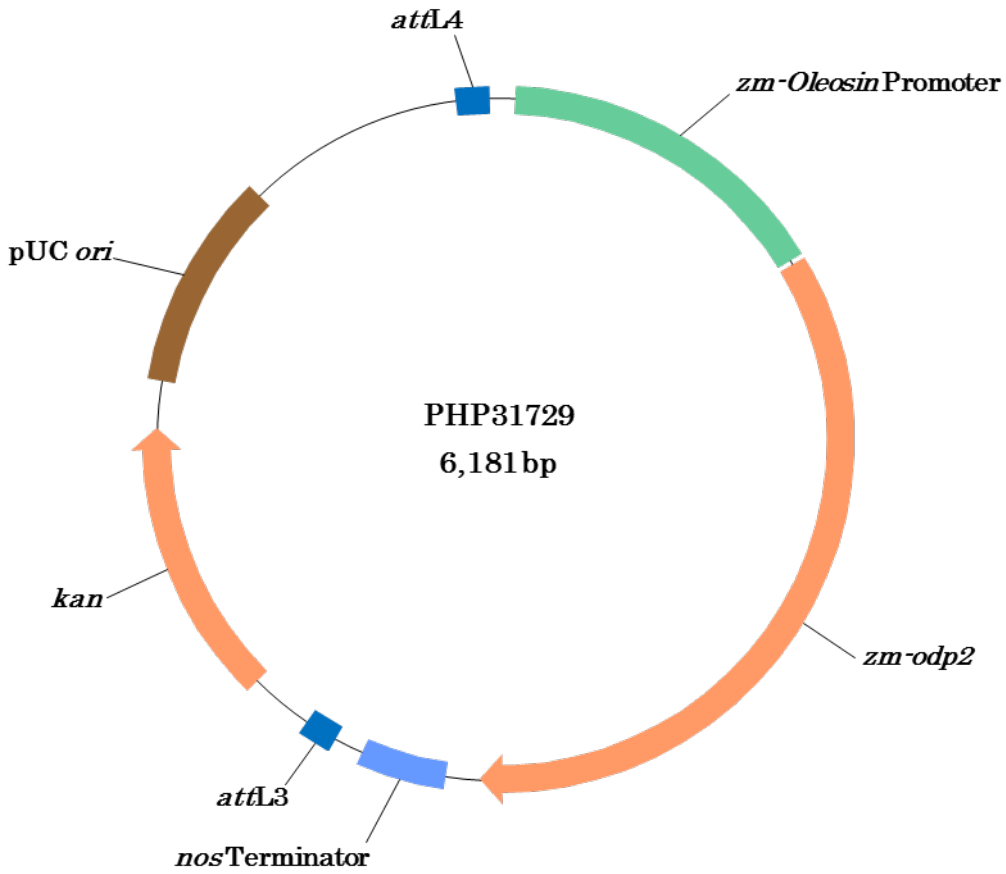

Figure 4. Schematic Diagram of Plasmid PHP31729
Schematic diagram of plasmid PHP31729 containing the *zm-odp2* gene cassette. The plasmid size is 6,181 bp. PHP31729 was used to enhance transformation and plant regeneration but was not incorporated into the maize genome.

III-A.1.2. Site-Specific Integration Gene Cassettes in the T-DNA of Binary Plasmid PHP74643

The *zm-wus2* gene cassette contains the maize *Wuschel2* (*wus2*) gene (Mayer et al., 1998) encoding the WUS protein. The expressed WUS protein enhances tissue regeneration during transformation (Lowe et al., 2016). The WUS protein is 302 amino acids in length and has a molecular weight of approximately 31 kDa. Expression of the *wus2* gene is controlled by the promoter from the *Agrobacterium tumefaciens* Ti plasmid nopaline synthase (*nos*) gene (Depicker et al., 1982), in conjunction with the presence of the terminator region from the potato (*Solanum tuberosum*) proteinase inhibitor II (*pin*II) gene (An et al., 1989; Keil et al., 1986).

The *zm-odp2* gene cassette contains the maize ovule development protein 2 (*odp2*) gene (GenBank accession XM008676474) encoding the ODP2 protein. The expressed ODP2 protein enhances the regeneration of maize plants from tissue culture after transformation (United States Patent 8420893). The ODP2 protein is 710 amino acids in length and has a molecular weight of approximately 74 kDa. Expression of the *odp2* gene is controlled by the promoter region from the maize ubiquitin gene 1 (*ubi*ZM1) including the 5' untranslated region (5' UTR) and intron (Christensen et al., 1992). The terminator for the *odp2* gene is a second copy of the *pin*II terminator. An additional terminator is present between the second and third cassettes: the terminator region from the maize 19-kDa zein (Z19) gene (GenBank accession KX247647; Dong et al., 2016). This additional terminator element is intended to prevent any potential transcriptional interference with the downstream cassettes. Transcriptional interference is defined as the transcriptional suppression of one gene on another when both are in close proximity (Shearwin et al., 2005). The placement of one or multiple transcriptional terminators between gene cassettes has been shown to reduce the occurrence of transcriptional interference (Greger et al., 1998).

The *mo-Flp* gene cassette contains maize-optimized exon 1 and exon 2 of the flippase (*Flp*) gene (Dymecki, 1996) from *Saccharomyces cerevisiae,* separated by an intron region from the potato *LS1* (*st-LS1*) gene (Eckes et al., 1986). The expressed FLP protein facilitates site specific recombination during transformation. The FLP protein is 423 amino acids in length and has a molecular weight of approximately 49 kDa. Expression of the *mo-Flp* gene is controlled by a second copy of the *ubi*ZM1 promoter, the 5' UTR and intron, in conjunction with a third copy of the *pin*II terminator.

The *DsRed2* gene cassette contains a modified red fluorescent protein (*DsRed2*) gene from *Discosoma sp.* (Wasson-Blader, 2001), in which an internal *Bst*E II restriction site was removed from the original *DsRed2* gene without altering the amino acid sequence of the expressed protein. The tissue-specific expression of the DsRed2 protein in the aleurone layer of the maize seed produces a red coloration in seeds that contain the DNA insertion, allowing for

differentiation during seed sorting. The DsRed2 protein is 225 amino acids in length and has a molecular weight of approximately 26 kDa. Expression of the *DsRed2* gene is controlled by the 35S enhancer region from the cauliflower mosaic virus genome (CaMV 35S enhancer) (Franck et al., 1980; Kay et al., 1987) and the promoter region from the barley (*Hordeum vulgare*) lipid transfer protein (*Ltp2*) gene (Kalla et al., 1994) which provides aleurone-specific transcription of the *DsRed2* gene. The terminator for the *DsRed2* gene is the 35S terminator region from the cauliflower mosaic virus genome (CaMV 35S terminator) (Franck et al., 1980; Guilley et al., 1982). An additional copy of the CaMV 35S terminator present between the fourth and fifth cassettes is intended to prevent transcriptional interference between cassettes.

III-A.1.3. Insertion of PHP74643 T-DNA

The second transformation step used to create DP23211 maize utilized *Agrobacterium*-mediated transformation with plasmid PHP74643 to transport the PHP74643 T-DNA into the plant cell nucleus; however, the T-DNA did not integrate into the genome. Rather, the FLP recombinase encoded in the T-DNA (outside of the FRT1 and FRT87 sites) exchanged the intended trait gene cassettes for the *npt*II cassette to result in the intended insertion. Table 2 lists the relevant genetic elements within the PHP74643 T-DNA and indicates whether they are present in the final event DP23211.

The zm-SEQ9 and zm-SEQ8 sequences are the continuous, endogenous sequences in the maize genome; the targeting of the landing pad was designed to insert the DP23211 sequence between the endogenous zm-SEQ9 and zm-SEQ8 sequences in the first transformation. Identical zm-SEQ9 and zm-SEQ8 sequences in the PHP56614 plasmid were used to provide sequence homology between the target site and the plasmid which allows a homologous recombination to occur when I-CREI endonuclease enzyme produces a double-strand break between the endogenous zm-SEQ9 and zmSEQ8 sequences in the maize genome. Thus, the landing pad between the zm-SEQ9 and zm-SEQ8 sequences in the PHP56614 plasmid was introduced into the maize genome at the target site between the endogenous zm-SEQ9 and zm-SEQ8 sequences.

Following transformation, *zm-wus2*, *zm-odp2*, mo-*Flp*, and *DsRed2* genes are transiently expressed without integration into the maize genome. The FLP recombinase exchanges the *npt*II cassette for the *pmi*, *mo-pat*, DvSSJ1, and *ipd072Aa* cassettes from PHP74643 T-DNA region, while the expression of WUS2 and ODP2 allows for improved regeneration of maize plants from the transformation process and the DsRed2 protein provides visual evidence to detect the undesired integration of the T-DNA in the genome.

After transformation, regeneration of maize plants, and molecular characterization by SbS™, a maize line with the intended DNA insertion and no unintended DNA sequence inserts was selected for further characterization and advanced in the event development process. A

schematic overview of the overall transformation and event development process for DP23211 maize is provided in Figure 8.

Table 2. Summary of Genetic Elements in PHP74643 T-DNA and Presence in DP23211 Maize

Genetic element	Description	Present in DP23211 Maize
Right Border	T-DNA right border	No
zm-wus2	Developmental gene for regeneration	No
zm-odp2	Developmental gene for regeneration	No
mo-Flp	Maize-optimized flippase recombinase gene	No
DsRed2	Red fluorescent protein gene	No
FRT1	Flippase recombination target site	Yes
pmi	Phosphomannose isomerase gene	Yes
mo-pat	Maize-optimized phosphinothricin acetyltransferase gene	Yes
*Lox*P	Cre recombination site	Yes
DvSSJ1	Fragment of the smooth septate junction protein 1 (*dvssj1*) gene from WCR	Yes
ipd072Aa	Insect protection protein gene	Yes
FRT87	Flippase recombination target site	Yes
Left Border	T-DNA left border	No

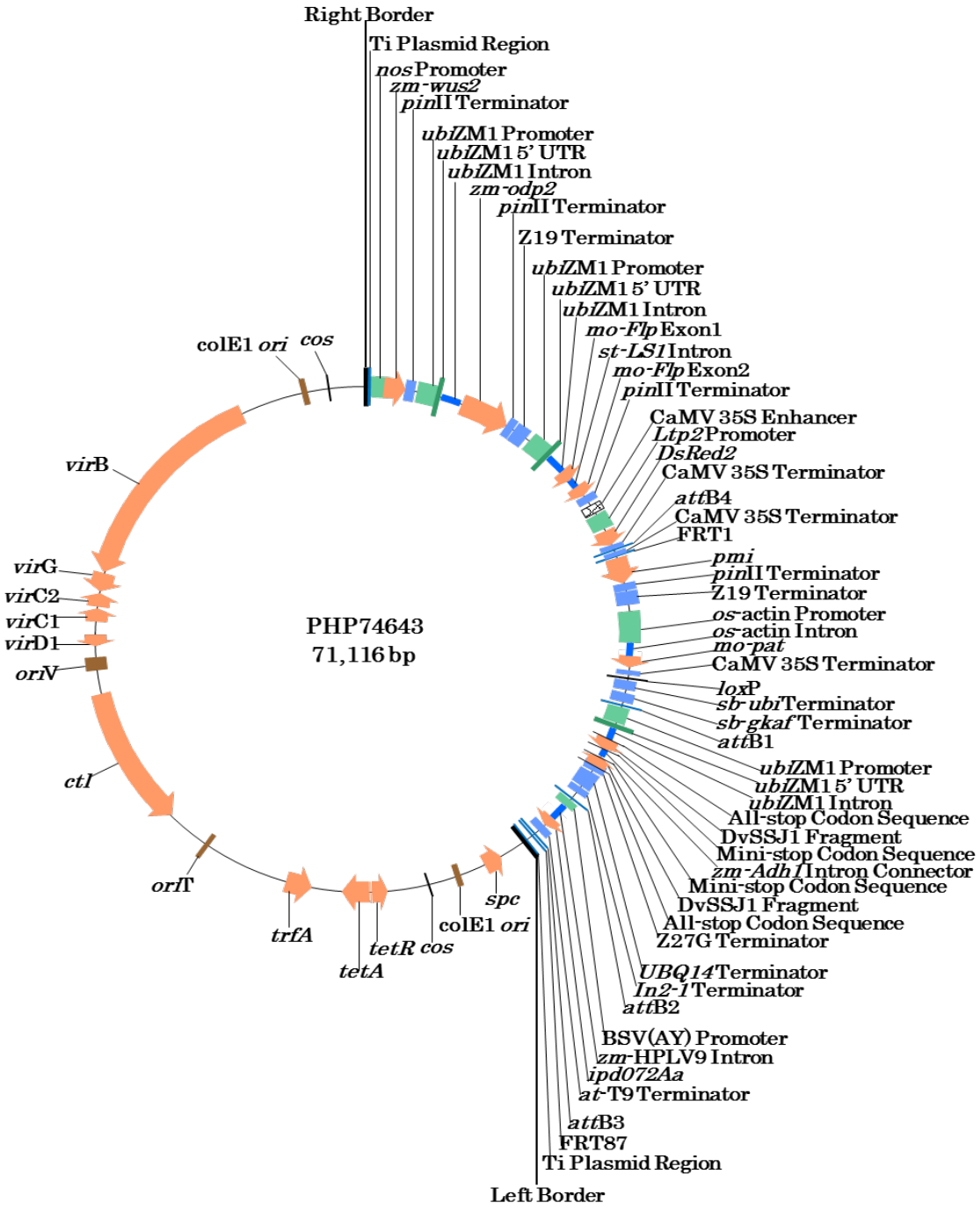

Figure 5. **PHP74643 Plasmid Map**

Schematic diagram of plasmid PHP74643 containing the DvSSJ1 fragment cassette and the *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes intended for incorporation into the maize genome and the *zm-wus2*, *zm-odp2*, *mo-Flp*, and *DsRed2* gene cassettes not intended for incorporation into the maize genome. The size of plasmid PHP74643 is 71,116 bp.

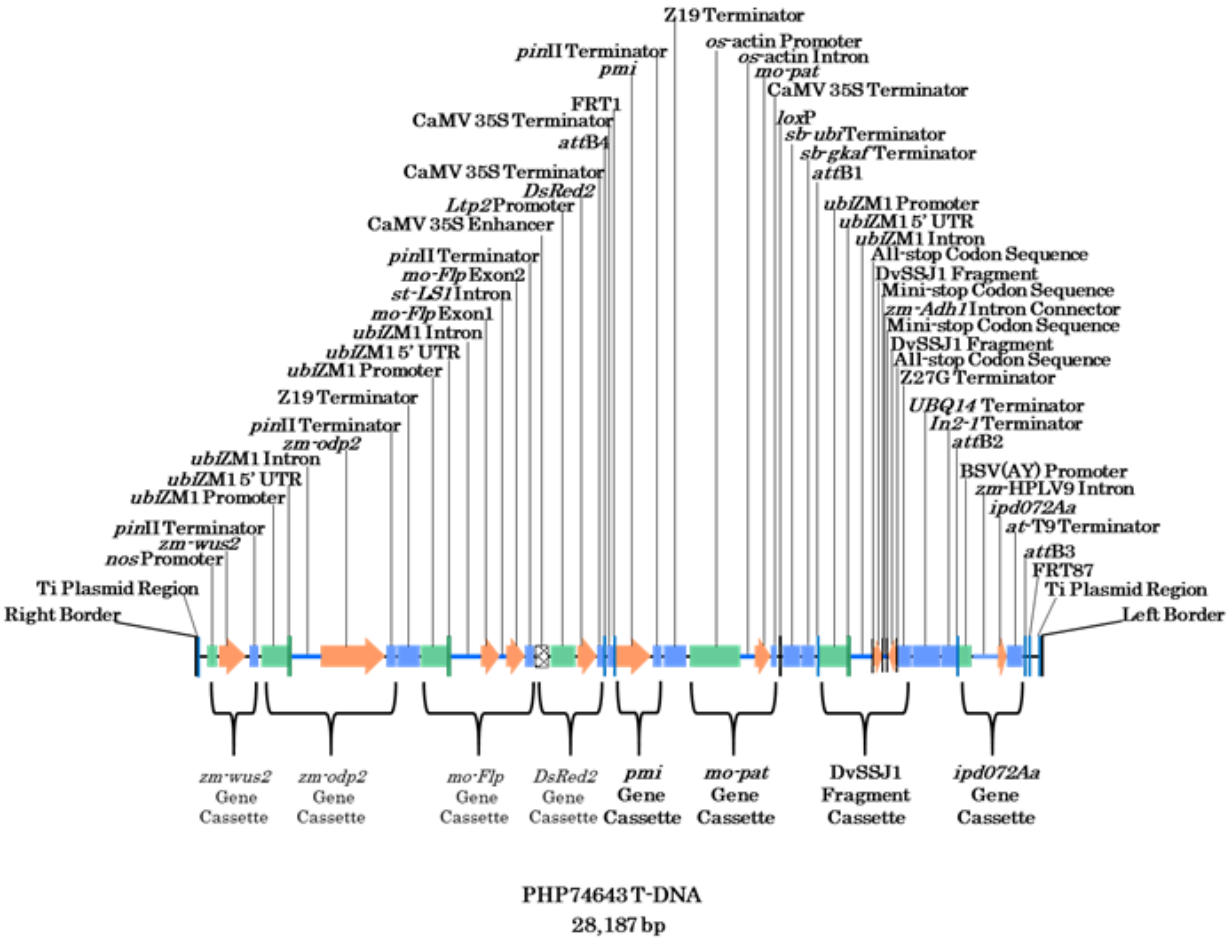

Figure 6. **Schematic Diagram of the T-DNA Region from Plasmid PHP74643**

Schematic diagram of the T-DNA region of plasmid PHP74643 indicating the DvSSJ1 fragment cassette and the *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes intended for incorporation into the maize genome and the *zm-wus2*, *zm-odp2*, *mo-Flp*, and *DsRed2* gene cassettes not intended for incorporation into the maize genome, defined as the fragment from 1 bp – 28,187 bp. The size of the T-DNA is 28,187 bp.

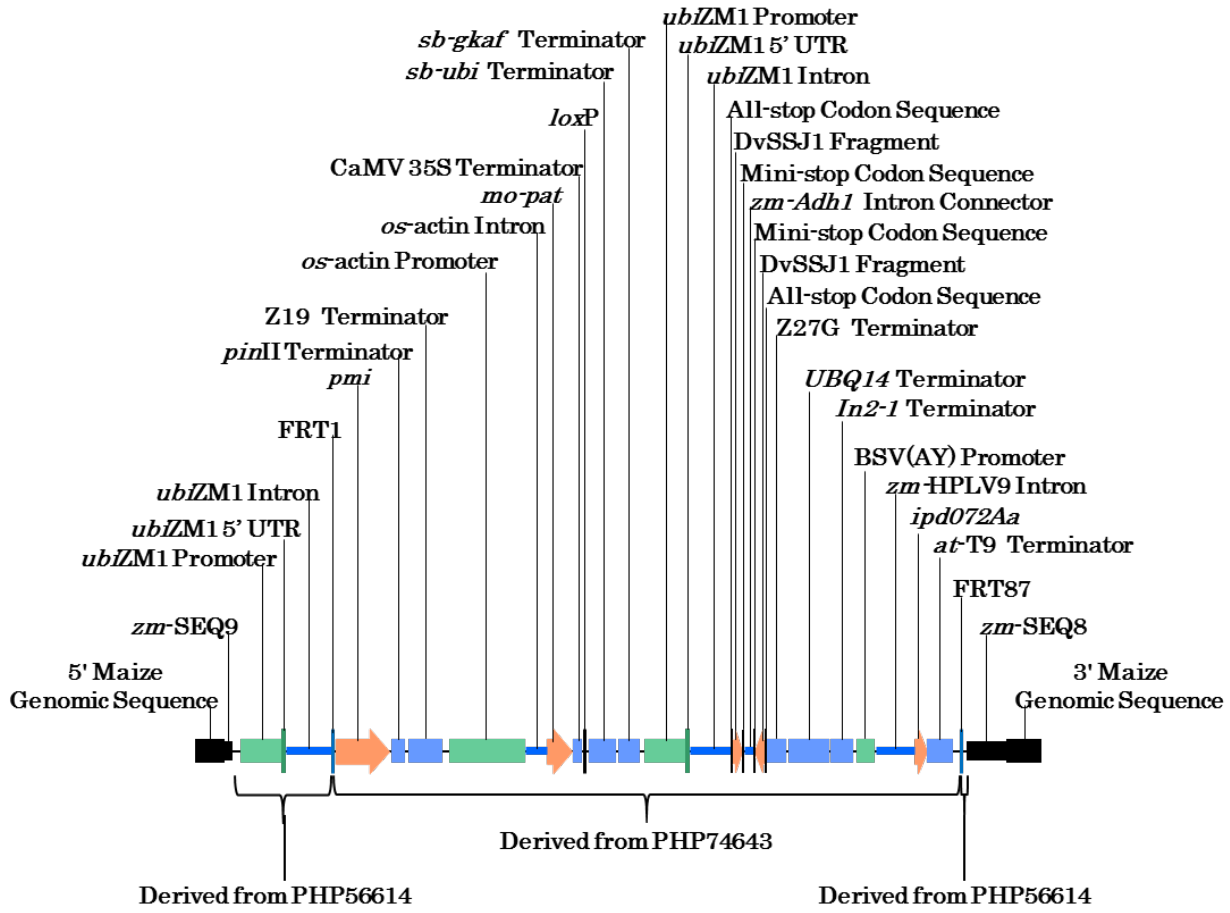

Figure 7. Map of the Intended DP23211 Insertion

Schematic map of the insertion intended to be present (bracketed regions) in the DP23211 maize genome following SSI at the FRT1 and FRT87 sites. The size of the intended insertion is 16,176 bp and it includes sequences from PHP74643 (Figure 5) and PHP56614 (Figure 1 and Figure 2). The flanking maize genomic regions are represented by horizontal black bars. Although *zm*-SEQ9 and *zm*-SEQ8 are present in PHP56614, they are derived from the maize genome and appear in their native context in the chromosome, so are considered to be parts of the flanking maize genome and are not included in the intended insertion.

III-A.1.4. Trait and Marker Gene Cassettes in the T-DNA of Binary Plasmid PHP74643

The *pmi* gene cassette contains the phosphomannose isomerase (*pmi*) gene from *Escherichia coli* (Negrotto et al., 2000). The expressed PMI protein in plant tissue serves as a selectable marker during transformation which allows for tissue growth using mannose as the carbon source. The PMI protein is 391 amino acids in length and has a molecular weight of approximately 43 kDa. As present in the T-DNA region of PHP74643, the *pmi* gene lacks a promoter, but its location next to the flippase recombination target site, FRT1, allows post-recombination expression by an appropriately-placed promoter. The terminator for the *pmi* gene is a fourth copy of the *pin*II terminator. An additional Z19 terminator present between the fifth and sixth cassettes is intended to prevent transcriptional interference between cassettes.

The *mo-pat* gene cassette contains a maize-optimized version of the phosphinothricin acetyl transferase gene (*mo-pat*) from *Streptomyces viridochromogenes* (Wohlleben et al., 1988) encoding the PAT protein. The expressed PAT protein confers tolerance to phosphinothricin. The PAT protein is 183 amino acids in length and has a molecular weight of approximately 21 kDa. Expression of the *mo-pat* gene is controlled by the promoter and intron region of the rice (*Oryza sativa*) actin (*os*-actin) gene (GenBank accession CP018159; GenBank accession EU155408.1), in conjunction with a third copy of the CaMV35S terminator. Two additional terminators are present between the sixth and seventh cassettes to prevent transcriptional interference: the terminator regions from the sorghum (*Sorghum bicolor*) ubiquitin (*sb-ubi*) gene (Phytozome gene ID Sobic.004G049900.1) and γ-kafarin (*sb-gkaf*) gene (de Freitas et al., 1994), respectively.

The DvSSJ1 fragment cassette is expressed as a transcript to form in an inverted repeat configuration that contains two RNA fragments of the smooth septate junction protein 1 (*dvssj1*) gene matching WCR (Hu et al., 2016) separated by an intron connector sequence derived from the intron 1 region of the maize alcohol dehydrogenase (*zm-Adh1*) gene (Dennis et al., 1984). Each of the two DvSSJ1 fragments is flanked by stop codon sequences designed to terminate translation through the site. The transcription product of this cassette, DvSSJ1 dsRNA, is intended to downregulate the expression of the DvSSJ1 protein in the mid-gut of WCR via RNAi. Expression of the DvSSJ1 fragment is controlled by a third copy of the *ubi*ZM1 promoter, the 5' UTR, and intron, in conjunction with the terminator region from the maize W64 line 27-kDa gamma zein (Z27G) gene (Das et al., 1991; Liu et al., 2016). Two additional terminators are present between the DvSSJ1 fragment cassette and the *ipd072Aa* gene cassette to prevent transcriptional interference: the terminator region from the *Arabidopsis thaliana* ubiquitin 14 (*UBQ*14*)* gene (Callis et al., 1995) and the terminator region from the maize *In2-1* gene (Hershey and Stoner, 1991).

The *ipd072Aa* gene cassette contains the insecticidal protein gene, *ipd072Aa,* from *Pseudomonas chlororaphis* (Schellenberger et al., 2016). The expressed IPD072Aa protein in plants is effective

against certain coleopteran pests by causing disruption of the midgut epithelium. The IPD072Aa protein is 86 amino acids in length and has a molecular weight of approximately 10 kDa. Expression of the *ipd072Aa* gene is controlled by the promoter region from the banana streak virus of acuminata Yunnan strain (BSV (AY)) (GenBank accession DQ092436.1; Zhuang et al., 2011) and the intron region from the maize ortholog of a rice (*Oryza sativa*) hypothetical protein (*zm*-HPLV9), in conjunction with the terminator region from the *Arabidopsis thaliana at*-T9 gene (GenBank accession NM_001202984; Salanoubat et al., 2000).

The PHP74643 T-DNA contains two Flp recombinase target sequences, FRT1 and FRT87 sites (Proteau et al., 1986; Tao et al., 2007, respectively), as well as one *lox*P (Dale and Ow, 1990) and four *attB* recombination sites (Cheo et al., 2004; Hartley et al., 2000; Katzen, 2007).

III.A.2. Selection of Event DP23211

Plants that were regenerated from transformation and tissue culture (designated T0 plants) were selected for further characterization. Refer to Figure 8 for a schematic overview of the transformation and event development process for DP23211 maize.

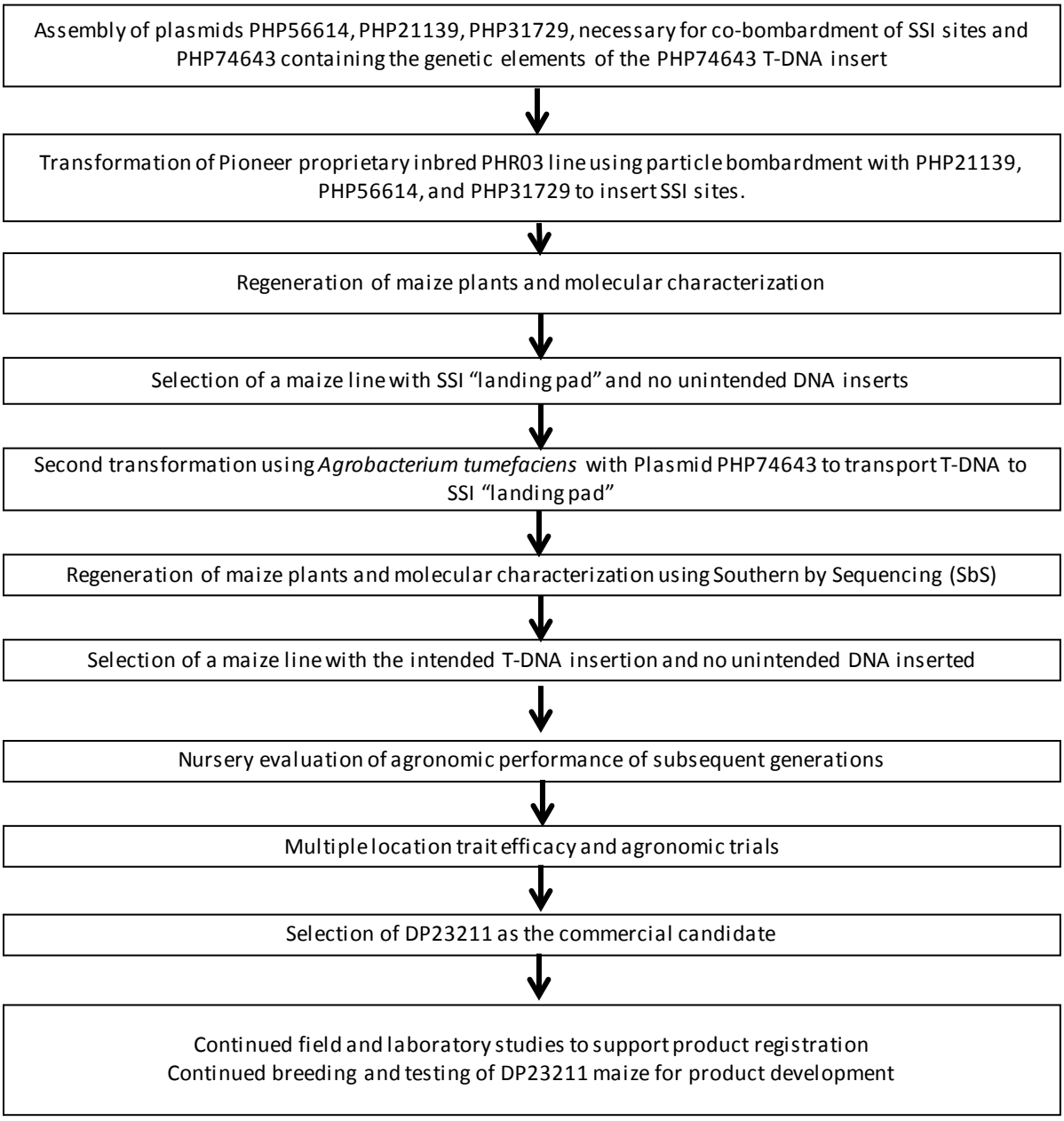

Figure 8. Event Development Process for DP23211 Maize

III.A.3. Breeding Method Used to Develop DP23211 Maize

The subsequent breeding of DP23211 maize proceeded as indicated in Figure 9 to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial maize lines. Table 3 provides the generations used for each characterization study.

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Figure 9. **Breeding Diagram for DP23211 Maize and Generations Used for Analysis**
The breeding steps to produce the generations used for characterization, assessment, and the development of commercial lines are shown schematically. Pioneer proprietary inbred PHR03 was used for transformation to produce DP23211 maize.

Table 3. Generations and Comparators Used for Analysis of DP23211 Maize

Analysis	Seed Generation(s) Used	Comparators
Copy Numbers, Intactness, and Backbone by SbS	T1	PHR03
Intactness and Stability by Southern Blot	T1, T2, T3, T4, T5	PHR03
Composition Analysis	F1 (PHEJW/PHR03)	PHEJW/PHR03
Mendelian Inheritance	BC1F1 (PH1V5T), BC2F1, T1, T5, BC1F1 (PH2SRH)	N/A
Agronomic Evaluation	F1 (PHEJW/PHR03)	PHEJW/PHR03

III.A.4. Selection of Comparators for DP23211 Maize

For the characterization of DP23211 maize, Pioneer proprietary maize F1 hybrid (PHEJW/PHR03) and inbred line (PHR03) were used as experimental controls (Table 3). The control lines selected are non-genetically engineered (non-GE) and represent the genetics of the maize lines used to produce the DP23211 maize generations used in analysis (Figure 9).

In addition, conventionally-bred (conventional) non-GE maize hybrid lines (*i.e.*, reference lines), were used to obtain tolerance intervals for compositional analyses. These maize hybrids were chosen to represent a wide range of conventional non-GE varieties that could be planted commercially. These tolerance intervals help represent the normal range of variation of the maize crop for compositional analytes and further helped to determine the comparability of DP23211 maize to conventional non-GE maize.

IV. Donor Genes and Regulatory Sequences in DP23211 Maize

IV-A. DNA Used in Transformation

DP23211 maize was produced by *Agrobacterium*-mediated transformation with plasmid PHP74643. (Figure 5, Table 4). The T-DNA insert in DP23211 maize (Figure 6) contains the *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes and the DvSSJ1 fragment cassette. A summary of the genetic elements and their position in the T-DNA region of PHP74643 is given in Table 5.

Table 4. Description of the Genetic Elements in Plasmid PHP74643

Region	Location on Plasmid (bp to bp)	Genetic Element	Size (bp)	Description
T-DNA	1 – 28,187		28,187	See Table 5 for information on the elements in this region
Plasmid Construct	28,188 – 53,071	Includes Elements Below	24,884	DNA from various sources for plasmid construction and plasmid replication
	29,363 – 30,151 (complementary)	*spc*	789	Spectinomycin resistance gene from bacteria (Fling et al., 1985)
	31,274 – 31,643 (complementary)	colE1 *ori*	370	Origin of replication region from *Escherichia coli* (Tomizawa et al., 1977)
	32,737 – 32,750	*cos*	14	Cohesive ends from lambda bacteriophage DNA (Komari et al., 1996)
	34,455 – 35,105 (complementary)	*tetR*	651	Tetracycline resistance regulation gene from bacteria (Komari et al., 1996)
	35,211 – 36,410	*tetA*	1,200	Tetracycline resistance gene from bacteria (Komari et al., 1996)
	37,683 – 38,831 (complementary)	*trfA*	1,149	Trans-acting replication gene from bacteria (Komari et al., 1996)
	42,613 – 42,979 (complementary)	*ori*T	367	Origin of transfer region from bacteria (Komari et al., 1996)
	44,596 – 50,866 (complementary)	*ctl*	6,271	Central control operon region from bacteria (Komari et al., 1996)
	51,874 – 52,584 (complementary)	*ori*V	711	Origin of replication region from bacteria (Komari et al., 1996)
Ti Plasmid Backbone	53,072 – 67,888	Includes Elements Below	14,817	Virulence (*vir*) gene region and intergenic regions from Ti plasmid of *Agrobacterium tumefaciens* (Komari et al., 1996)
	53,385 – 53,828 (complementary)	*vir*D1	444	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	54,097 – 54791	*vir*C1	695	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	54,794 – 55,402	*vir*C2	609	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	55,513 – 56,316 (complementary)	*vir*G	804	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	56,448 – 65,883 (complementary)	*vir*B	9,436	Virulence operon region from *Agrobacterium tumefaciens* important for T-DNA insertion into genome

Table 4. Description of the Genetic Elements in Plasmid PHP74643 (continued)

Region	Location on plasmid (bp to bp)	Genetic Element	Size (bp)	Description
Plasmid Construct	67,889 – 71,116	Includes Elements Below	3,228	DNA from various sources for plasmid construction and plasmid replication
	68,184 – 68,553 (complementary)	colE1 *ori*	370	Origin of replication region from *Escherichia coli* (Tomizawa et al., 1977)
	69,647 – 69,660	*cos*	14	Cohesive ends from lambda bacteriophage DNA (Komari et al., 1996)

Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP74643

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	1 – 25	Right Border (RB)	25	T-DNA Right Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)
	26 – 75	Ti Plasmid Region	50	Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)
	76 – 406	Intervening Sequence	331	DNA sequence used for cloning
zm-wus2 gene cassette	407 – 728	*nos* Promoter	322	Promoter region from the *Agrobacterium tumefaciens* Ti plasmid nopaline synthase gene (Depicker et al., 1982)
	729 – 781	Intervening Sequence	53	DNA sequence used for cloning
	782 – 1690	*zm-wus2*	909	*Wuschel 2* gene from *Zea mays* (Lowe et al., 2016; Mayer et al., 1998)
	1,691 – 1,802	Intervening Sequence	112	DNA sequence used for cloning
	1,803 – 2,113	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	2,114 – 2,175	Intervening Sequence	62	DNA sequence used for cloning
zm-odp2 gene cassette	2,176 – 3,075	*ubi*ZM1 Promoter	900	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	3,076 – 3,158	*ubi*ZM1 5' UTR	83	5' untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	3,159 – 4,171	*ubi*ZM1 Intron	1,013	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	4,172 – 4,189	Intervening Sequence	18	DNA sequence used for cloning
	4,190 – 6,322	*zm-odp2*	2,133	Ovule development protein 2 gene from *Zea mays* (U.S. Patent 8420893; GenBank accession XM008676474)
	6,323 – 6,390	Intervening Sequence	68	DNA sequence used for cloning
	6,391 – 6,701	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	6,702 – 6,717	Intervening Sequence	16	DNA sequence used for cloning

Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP74643 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	6,718 – 7,459	Z19 Terminator	742	Terminator region from the *Zea mays* 19-kDa zein gene (GenBank accession KX247647; Dong et al., 2016)
	7,460 – 7,480	Intervening Sequence	21	DNA sequence used for cloning
mo-Flp gene cassette	7,481 – 8,380	*ubi*ZM1 Promoter	900	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	8,381 – 8,463	*ubi*ZM1 5' UTR	83	5' untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	8,464 – 9,476	*ubi*ZM1 Intron	1,013	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	9,477 – 9,506	Intervening Sequence	30	DNA sequence used for cloning
	9,507 – 10,140	*mo-Flp* Exon1	634	Maize-optimized exon 1 of the flippase gene from *Saccharomyces cerevisiae* (Dymecki, 1996)
	10,141 – 10,329	*st-LS1* Intron	189	Intron region from the *Solanum tuberosum* (potato) *LS1* gene (Eckes et al., 1986)
	10,330 – 10,967	*mo-Flp* Exon2	638	Maize-optimized exon 2 of the flippase gene from *Saccharomyces cerevisiae* (Dymecki, 1996)
	10,968 – 10,972	Intervening Sequence	5	DNA sequence used for cloning
	10,973 – 11,283	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	11,284 – 11,296	Intervening Sequence	13	DNA sequence used for cloning
DsRed2 gene cassette	11,297 – 11,771	CaMV 35S Enhancer	475	35S enhancer region from the cauliflower mosaic virus genome (Franck et al., 1980; Kay et al., 1987)
	11,772 – 11,804	Intervening Sequence	33	DNA sequence used for cloning
	11,805 – 12,650	*Ltp2* Promoter	846	Promoter region from the *Hordeum vulgare* (barley) aleurone-specific lipid transfer protein gene (Kalla et al., 1994)
	12,651 – 12,694	Intervening Sequence	44	DNA sequence used for cloning
	12,695 – 13,372	*DsRed2*	678	Modified red fluorescent protein gene from *Discosoma sp* (Wasson-Blader, 2001) with internal *Bst*E II restriction site removed

Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP74643 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
DsRed2 gene cassette (cont.)	13,373 – 13,376	Intervening Sequence	4	DNA sequence used for cloning
	13,377 – 13,570	CaMV 35S Terminator	194	35S terminator region from the cauliflower mosaic virus genome (Franck et al., 1980; Guilley et al., 1982)
	13,571 – 13,596	Intervening Sequence	26	DNA sequence used for cloning
	13,597 – 13,617	*att*B4	21	Bacteriophage lambda integrase recombination site (Cheo et al., 2004)
	13,618 – 13,696	Intervening Sequence	79	DNA sequence used for cloning
	13,697 – 13,890	CaMV 35S Terminator	194	35S terminator region from the cauliflower mosaic virus genome (Franck et al., 1980; Guilley et al., 1982)
	13,891 – 13,905	Intervening Sequence	15	DNA sequence used for cloning
	13,906 – 13,953	FRT1	48	Flippase recombination target site from *Saccharomyces cerevisiae* (Proteau et al., 1986)
	13,954 – 13,971	Intervening Sequence	18	DNA sequence used for cloning
pmi gene cassette	13,972 – 15,187	*pmi*	1,216	Phosphomannose isomerase gene from *Escherichia coli* including 5' and 3' untranslated regions (UTR) (Negrotto et al., 2000) as described below: 5' UTR at bp 13,972-13,975 (4 bp long) Coding sequence at bp 13,976-15,151 (1,176 bp long) 3' UTR at bp 15,152-15,187 (36 bp long)
	15,188 – 15,197	Intervening Sequence	10	DNA sequence used for cloning
	15,198 – 15,508	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	15,509 – 15,518	Intervening Sequence	10	DNA sequence used for cloning
	15,519 – 16,260	Z19 Terminator	742	Terminator region from the *Zea mays* 19-kDa zein gene (GenBank accession KX247647; Dong et al., 2016)

Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP74643 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	16,261 – 16,463	Intervening Sequence	203	DNA sequence used for cloning
mo-pat gene cassette	16,464 – 18,145	*os*-actin Promoter	1,682	Promoter region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159; GenBank accession EU155408.1)
	18,146 – 18,614	*os*-actin Intron	469	Intron region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159; GenBank accession EU155408.1)
	18,615 – 18,629	Intervening Sequence	15	DNA sequence used for cloning
	18,630 – 19,181	*mo-pat*	552	Maize-optimized phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes* (Wohlleben et al., 1988)
	19,182 – 19,199	Intervening Sequence	18	DNA sequence used for cloning
	19,200 – 19,393	CaMV 35S Terminator	194	35S terminator region from the cauliflower mosaic virus genome (Franck et al., 1980; Guilley et al., 1982)
	19,394 – 19,414	Intervening Sequence	21	DNA sequence used for cloning
	19,415 – 19,448	*lox*P	34	Bacteriophage P1 recombination site recognized by Cre recombinase (Dale and Ow, 1990)
	19,449 – 19,544	Intervening Sequence	96	DNA sequence used for cloning
	19,545 – 20,128	*sb-ubi* Terminator	584	Terminator region from the *Sorghum bicolor* (sorghum) ubiquitin gene (Phytozome gene ID Sobic.004G049900.1)
	20,129 – 20,169	Intervening Sequence	41	DNA sequence used for cloning
	20,170 – 20,633	*sb-gkaf* Terminator	464	Terminator region from the *Sorghum bicolor* (sorghum) γ-kafarin gene (de Freitas et al., 1994)
	20,634 – 20,666	Intervening Sequence	33	DNA sequence used for cloning
	20,667 – 20,690	*att*B1	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning system (Hartley et al., 2000; Katzen, 2007)

Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP74643 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	20,691 – 20,777	Intervening Sequence	87	DNA sequence used for cloning
DvSSJ1 fragment cassette	20,778 – 21,677	*ubi*ZM1 Promoter	900	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	21,678 – 21,760	*ubi*ZM1 5' UTR	83	5’ untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	21,761 – 22,773	*ubi*ZM1 Intron	1,013	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	22,774 – 22,798	Intervening Sequence	25	DNA sequence used for cloning
	22,799 – 22,812	All-stop Codon Sequence	14	DNA sequence containing stop codons to terminate translation in all six reading frames through the site
	22,813 – 23,022	DvSSJ1 Fragment	210	Fragment of the smooth septate junction protein 1 gene from *Diabrotica virgifera* (Western corn rootworm; Hu et al., 2016)
	23,023 – 23,030	Mini-stop Codon Sequence	8	DNA sequence containing stop codons to terminate translation in designated reading frames through the site
	23,031 – 23,041	Intervening Sequence	11	DNA sequence used for cloning
	23,042 – 23,147	*zm-Adh1* Intron Connector	106	Connector sequence derived from the intron 1 region of the *Zea mays* alcohol dehydrogenase gene (Dennis et al., 1984)
	23,148 – 23,156	Intervening Sequence	9	DNA sequence used for cloning
	23,157 – 23,164 (complementary)	Mini-stop Codon Sequence	8	DNA sequence containing stop codons to terminate translation in designated reading frames through the site
	23,165 – 23,374 (complementary)	DvSSJ1 Fragment	210	Fragment of the smooth septate junction protein 1 gene from *Diabrotica virgifera* (Western corn rootworm; Hu et al., 2016)
	23,375 – 23,388 (complementary)	All-stop Codon Sequence	14	DNA sequence containing stop codons to terminate translation in all six reading frames through the site
	23,389 – 23,408	Intervening Sequence	20	DNA sequence used for cloning
	23,409 – 23,888	Z27G Terminator	480	Terminator region from the *Zea mays* W64 line 27-kDa gamma zein gene (Das et al., 1991; Liu et al., 2016)

Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP74643 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	23,889 – 23,894	Intervening Sequence	6	DNA sequence used for cloning
	23,895 – 24,796	*UBQ14* Terminator	902	Terminator region from the *Arabidopsis thaliana* ubiquitin 14 gene (Callis et al., 1995)
	24,797 – 24,802	Intervening Sequence	6	DNA sequence used for cloning
	24,803 – 25,296	*In2-1* Terminator	494	Terminator region from the *Zea mays In2-1* gene (Hershey and Stoner, 1991)
	25,297 – 25,353	Intervening Sequence	57	DNA sequence used for cloning
	25,354 – 25,377	*att*B2	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning System (Hartley et al., 2000; Katzen, 2007)
	25,378 – 25,414	Intervening Sequence	37	DNA sequence used for cloning
ipd072Aa gene cassette	25,415 – 25,828	BSV(AY) Promoter	414	Promoter region from the banana streak virus (acuminata Yunnan strain) genome (GenBank accession DQ092436.1; Zhuang et al., 2011)
	25,829 – 25,847	Intervening Sequence	19	DNA sequence used for cloning
	25,848 – 26,703	*zm*-HPLV9 Intron	856	Intron region from the *Zea mays* predicted calmodulin 5 gene (Phytozome gene ID Zm00008a029682)
	26,704 – 26,712	Intervening Sequence	9	DNA sequence used for cloning
	26,713 – 26,973	*ipd072Aa*	261	Insecticidal protein gene from *Pseudomonas chlororaphis* (Schellenberger et al., 2016)
	26,974 – 26,979	Intervening Sequence	6	DNA sequence used for cloning
	26,980 – 27,552	*at*-T9 Terminator	573	Terminator region from an *Arabidopsis thaliana* putative gene of the mannose-binding protein superfamily (GenBank accession NM_001202984; Salanoubat et al., 2000)
	27,553 – 27,591	Intervening Sequence	39	DNA sequence used for cloning
	27,592 – 27,612 (complementary)	*att*B3	21	Bacteriophage lambda integrase recombination site (Cheo et al., 2004)
	27,613 – 27,733	Intervening Sequence	121	DNA sequence used for cloning

Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP74643 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	27,734 – 27,781	FRT87	48	Modified Flippase recombination target site derived from *Saccharomyces cerevisiae* (Tao et al., 2007)
	27,782 – 28,112	Intervening Sequence	331	DNA sequence used for cloning
	28,113 – 28,162	Ti Plasmid Region	50	Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)
	28,163 – 28,187	Left Border (LB)	25	T-DNA Left Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)

V. Molecular Characterization and Genetic Stability of DP23211 Maize

V-A. Molecular Analysis Overview

Molecular characterization of GE events determines the insertion copy number, integrity of the insertion, and absence of plasmid DNA unintended for integration. The inserted DNA is also evaluated over several generations of plants to confirm its stable Mendelian inheritance. DP23211 maize plants were characterized by a Next Generation Sequencing (NGS) method known as Southern-by-Sequencing (SbS™ technology, hereafter referred to as SbS) method to determine the number of insertions within the plant genome, insertion integrity, and to confirm the absence of plasmid backbone sequences. Southern blot analysis was performed to confirm stable genetic inheritance of the inserted *DvSSJ1* fragment cassette and *ipd072Aa*, *mo-pat* and *pmi* gene cassettes.

Based on the SbS analysis described below, it was determined that a single, intact PHP74643 T-DNA was inserted into the genome of DP23211 maize, and that no unintended insertions or plasmid backbone sequences are present in its genome. In addition, Southern blot analysis and Mendelian segregation analysis across five breeding generations confirmed the stable genetic inheritance of the DNA insertion in DP23211 maize.

V-B. Southern-by-Sequencing (SbS) Analysis for Copy Number, Integrity, and Confirmation of the Absence of Vector Backbone Sequence

SbS analysis utilizes probe-based sequence capture, Next Generation Sequencing (NGS) techniques, and bioinformatics procedures to capture, sequence, and identify inserted DNA within the maize genome. By compiling a large number of unique sequencing reads and mapping them against the transformation plasmid and control maize genome, unique junctions due to inserted DNA are identified in the bioinformatics analysis and used to determine the number of insertions within the plant genome, verify insertion intactness, and confirm the absence of plasmid backbone sequences

The SbS technique (Figure 10) utilizes capture probes homologous to the transformation plasmid to isolate genomic DNA that hybridizes to the probe sequences (Zastrow-Hayes et al., 2015). Captured DNA is then sequenced using a Next Generation Sequencing (NGS) procedure and the results are analyzed using bioinformatics tools. During the analysis, junction reads are identified as those sequence reads where part of the read shows exact homology to the plasmid DNA sequence while the rest of the read does not match the contiguous plasmid. Junctions may occur between inserted DNA and genomic DNA, or between insertions of two plasmid-derived DNA sequences that are not contiguous in the transformation plasmid. Multiple sequence reads are generated for each junction and are compiled into a consensus sequence for the junction. By compiling a large number of unique sequencing reads and comparing them to the transformation

plasmid and control maize genome, unique junctions due to inserted DNA are identified. A unique junction is defined as one in which the plasmid-derived sequence and the adjacent sequence are the same across multiple reads, although the overall length of the multiple reads for that junction will vary due to the sequencing process. The number of unique junctions is related to the number of plasmid insertions present in the maize genome (for example, a single T-DNA insertion is expected to have two unique junctions). Detection of additional unique junctions beyond the two expected for a single insertion would indicate the presence of rearrangements or additional insertions derived from plasmid DNA. Absence of any junctions indicates there are no detectable insertions within the maize genome.

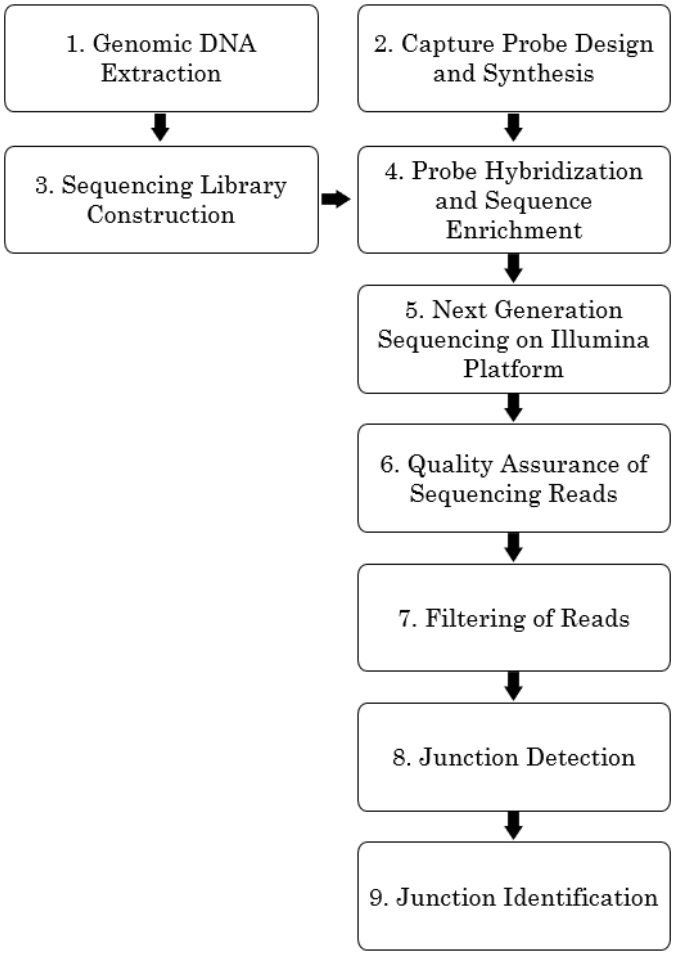


Figure 10. **Southern-by-Sequencing (SbS) Process Flow Diagram**

The T1 generation of DP23211 maize was analyzed by SbS, using full-coverage probes comprising the entire sequences of the trait plasmid PHP74643, the landing pad plasmid PHP56614, and the helper plasmids PHP21139 and PHP31729, to determine the insertion copy number and intactness and to confirm the absence of plasmid backbone sequences or unintended plasmid integration. SbS was also performed on control maize, and on positive control samples of each plasmid to confirm that the assay could reliably detect plasmid fragments spiked into control maize genomic DNA at a level equivalent to one copy of plasmid per genome copy. Based on the results obtained in this study, a schematic diagram of the DP23211 insertion was developed and is provided in Figure 11.

Several genetic elements in the positive control plasmids are derived from maize and thus the homologous elements in the PHR03 maize genome will be captured by the full-coverage probes used in the SbS analysis. These endogenous elements (*zm-wus2*, *ubi*ZM1 promoter, 5' UTR, and intron, *zm-odp2*, Z19 terminator, *zm-Adh1* intron connector, Z27G terminator, *In2-1* terminator, *zm*-HPLV9 intron, *zm*-SEQ9, *zm*-SEQ8, *In2-2* promoter, and *zm-Oleosin* promoter; (Table 6, Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, and Figure 6) will have sequencing reads in the SbS results due to the homologous elements in the PHR03 maize genome. However, if no junctions are detected, these sequencing reads only indicate the presence of the endogenous elements in their normal context of the maize genome and are not from inserted DNA.

SbS analysis results for the control maize are shown in Figure 12 and the positive control samples are presented in Figure 13. Example SbS results for one positive plant from the DP23211 maize T1 generation are presented in Figure 14. The SbS results for the remaining 9 plants tested are presented in Appendix 2. Methods and Results for Southern by Sequencing Analysis.

Sequencing reads were detected in the PHR03 control maize (Figure 12); however, coverage above background level (35x) was obtained only for the genetic elements derived from the maize genome. These sequence reads were due to capture and sequencing of these genetic elements in their normal context within the PHR03 control maize genome (Table 6). Variation in coverage of the endogenous elements is due to sequence variations between the PHR03 control maize and the maize varieties from which the genetic elements in the four plasmids were derived. No junctions were detected between plasmid sequences and the maize genome (Table 7), indicating that there are no plasmid DNA insertions in the control maize, and the sequence reads were solely due to the endogenous genetic elements present in the PHR03 control maize genome.

SbS analysis of the positive control samples resulted in sequence coverage across the entire length of each plasmid (Figure 13), indicating that the SbS assay utilizing the full-coverage probe library is sensitive enough to detect PHP74643, PHP56614, PHP21139, or PHP31729 sequences at a concentration equivalent to one copy of plasmid per copy of the maize genome. No junctions were detected between plasmid and genomic sequences (Table 7), indicating that the sequence

reads were due to either the spiked-in plasmid or the endogenous maize genetic elements that were detected in the control maize.

SbS analysis of the T1 generation of DP23211 maize resulted in three plants that contained the intended insertion (Table 6, Figure 14, and Appendix 2. Methods and Results for Southern by Sequencing Analysis, Figure 51., and Figure 52). Each of these plants contained two unique genome-insertion junctions, one at each end of the intended insertion, that were identical across the three plants. The 5′ junction starts with bp 1 of the intended insertion, derived from PHP56614 and PHP74643 (Table 6), and the insertion ends with the 3′ junction at bp 16,176 of the intended insertion. The number of sequence reads at the 5′ and 3′ junctions is provided in Table 7. There were no other junctions between PHP74643, PHP56614, PHP21139, or PHP31729 plasmid sequences and the maize genome detected in the plants, indicating that there are no additional plasmid-derived insertions present in DP23211 maize. Alignment of the reads from the positive plants to the four plasmid maps (Figure 14, and Appendix 2. Methods and Results for Southern by Sequencing Analysis, Figure 51, and Figure 52) shows coverage of the genetic elements found in the intended insertion, along with coverage of the endogenous elements in the plasmids that were not incorporated into the insertion (*zm-wus2*, *zm-odp2*, *In2-2* promoter, *zm-Oleosin* promoter, *zm*-SEQ9, and *zm*-SEQ8). Reads also aligned to the *pin*II terminator elements present outside of the intended insertion regions in PHP56614 and PHP74643 although these elements were not incorporated into the insertion. The NGS reads that aligned to these copies of the *pin*II terminator are from fragments containing the *pin*II terminator in the *pmi* cassette of the intended insertion; however, the reads from this single copy align to all copies of the *pin*II terminator in the plasmid maps. Similarly, reads aligned to the CaMV 35S terminator elements in the *DsRed2* cassette of PHP74643 due to the presence of an identical element in the *mo-pat* cassette of the intended insertion, and reads aligned to a portion of PHP31729 containing a Gateway™ *att* site element that matches a corresponding *att* site in the intended insertion.

There were no unexpected junctions between non-contiguous regions of the intended insertion identified, indicating that there are no rearrangements or truncations in the inserted DNA. Furthermore, there were no junctions between maize genome sequences and the backbone sequence of any of the plasmids involved in the production of DP23211 maize, demonstrating that no plasmid backbone sequences were incorporated into DP23211 maize.

Each of the seven DP23211 maize plants from the T1 generation that were determined to be negative for the DP23211 insertion yielded sequencing reads (Table 7, Appendix 2. Methods and Results for Southern by Sequencing Analysis, Figure 44, Figure 45, Figure 46, Figure 47, Figure 48, Figure 49, and Figure 50) that matched the reads in the control maize, indicating the reads were due to endogenous maize sequences. There were no junctions between plasmid sequences and

the maize genome detected in these plants, indicating that these plants did not contain any insertions derived from PHP74643, PHP56614, PHP21139, or PHP31729.

SbS analysis of the T1 generation of DP23211 maize demonstrated that there is a single, intact insertion of the intended insertion, derived from PHP56614 and the PHP74643 T-DNA, in DP23211 maize and that no additional insertions or plasmid backbone sequences are present in its genome.

Table 6. Maize Endogenous Elements in Plasmids and DP23211 Insertion

Number[a]	Name of Endogenous Element[b]	Present in Plasmid(s) or Insertion
1	*zm-wus2*	PHP21139, PHP74643
2	*ubi*ZM1 promoter, 5' UTR, and intron	PHP56614, PHP74643, DP23211 insertion
3	*zm-odp2*	PHP31729, PHP74643
4	Z19 terminator	PHP74643, DP23211 insertion
5	*zm-Adh1* intron connector	PHP74643, DP23211 insertion
6	Z27G terminator	PHP74643, DP23211 insertion
7	*In2-1* terminator	PHP21139, PHP74643, DP23211 insertion
8	*zm*-HPLV9 intron	PHP74643, DP23211 insertion
9	*zm*-SEQ9	PHP56614, DP23211 insertion[c]
10	*zm*-SEQ8	PHP56614, DP23211 insertion[c]
11	*In2-2* promoter	PHP21139
12	*zm-Oleosin* promoter	PHP31729

[a]Circled numbers found below linear construct maps in Figures 12-14 and Appendix 2 Figures 44-52.
[b]As shown in the plasmid and T-DNA maps in Figures 1 to 6 and the intended insertion map in Figure 7.
[c]As *zm*-SEQ9 and *zm*-SEQ8 are found in their native context in the genomic flanking regions, they are considered part of the flanking regions and not part of the DP23211 insertion.

Table 7. DP23211 Maize and Control Maize SbS Junction Reads

Sample Description	Supporting Reads at 5′ Junction[a]	Unique Reads at 5′ Junction[b]	Supporting Reads at 3′ Junction[c]	Unique Reads at 3′ Junction[d]	DP23211 Insertion
DP23211 Maize Plant ID 343210845	96	39	37	22	+
DP23211 Maize Plant ID 343210846	0	0	0	0	-
DP23211 Maize Plant ID 343210847	0	0	0	0	-
DP23211 Maize Plant ID 343210848	0	0	0	0	-
DP23211 Maize Plant ID 343210849	0	0	0	0	-
DP23211 Maize Plant ID 343210850	0	0	0	0	-
DP23211 Maize Plant ID 343210851	0	0	0	0	-
DP23211 Maize Plant ID 343210852	0	0	0	0	-
DP23211 Maize Plant ID 343210853	137	44	38	23	+
DP23211 Maize Plant ID 343210854	85	33	56	29	+
Control Maize[e]	0	0	0	0	-
PHP21139 Positive Control[e]	0	0	0	0	-
PHP31729 Positive Control[e]	0	0	0	0	-
PHP56614 Positive Control[e]	0	0	0	0	-
PHP74643 Positive Control[e]	0	0	0	0	-

[a] Total number of sequence reads across the 5′ junction of the DP23211 insertion.
[b] Unique sequence reads establishing the location of the 5′ genomic junction of the DP23211 insertion (Figure 11). Multiple identical NGS supporting reads are condensed into each unique read.
[c] Total number of sequence reads across the 3′ junction of the DP23211 insertion.
[d] Unique sequence reads establishing the location of the 3′ genomic junction of the DP23211 insertion (Figure 11). Multiple identical NGS supporting reads are condensed into each unique read.
[e] No genome-plasmid junctions were detected in either the control maize or positive control samples.

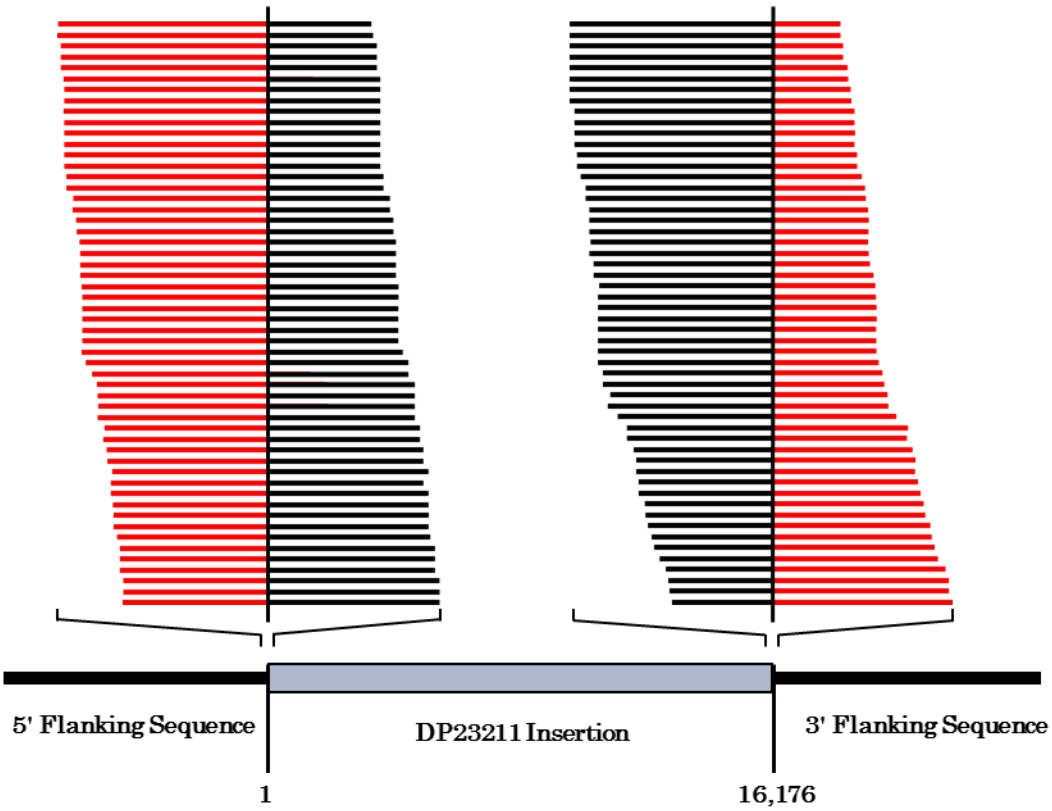
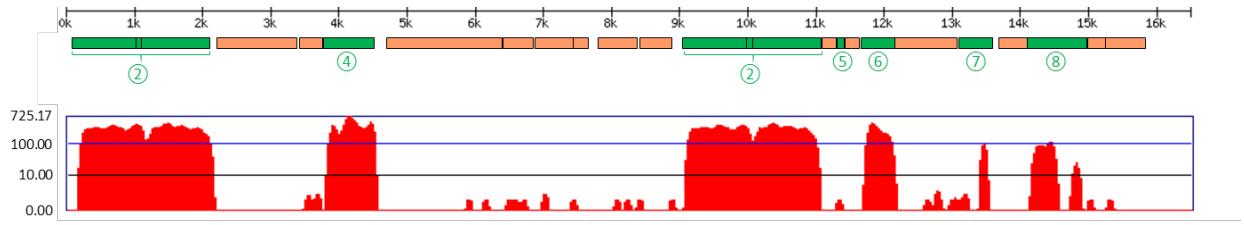


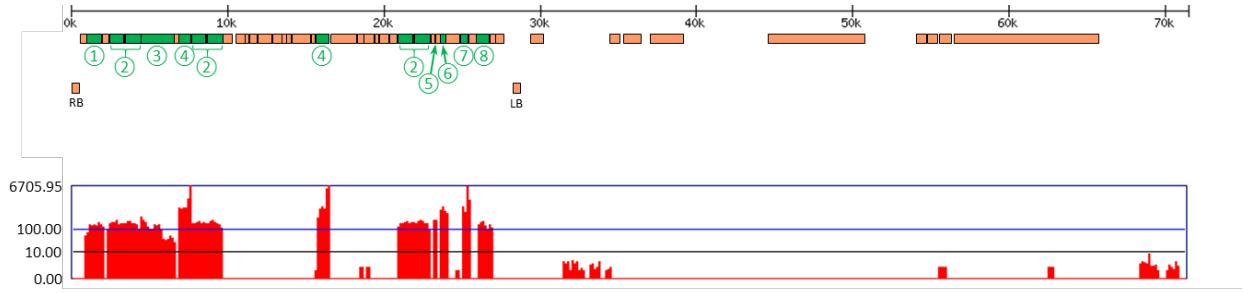
Figure 11. Map of the Insertion in DP23211 Maize

Schematic map of the DNA insertion in DP23211 maize based on the SbS analysis described. The flanking maize genomic regions, including *zm*-SEQ9 and *zm*-SEQ8, are indicated in the map by black bars. A single, intact copy of the intended insertion, derived from PHP56614 and PHP74643 and shown by the gray box, is integrated into the maize genome. Vertical lines show the locations of the two-unique genome-insertion junctions. The numbers below the map indicate the bp location of the junction nucleotide in reference to the sequence of the intended insertion (Figure 7). Representative individual sequencing reads across the junctions are shown as stacked lines above each junction (not to scale); red indicates genomic flanking sequence and black indicates T-DNA sequence within each read.

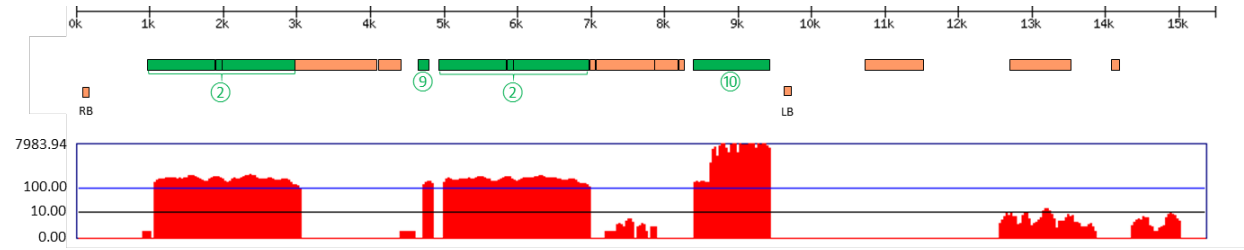
A. Alignment to Intended Insertion



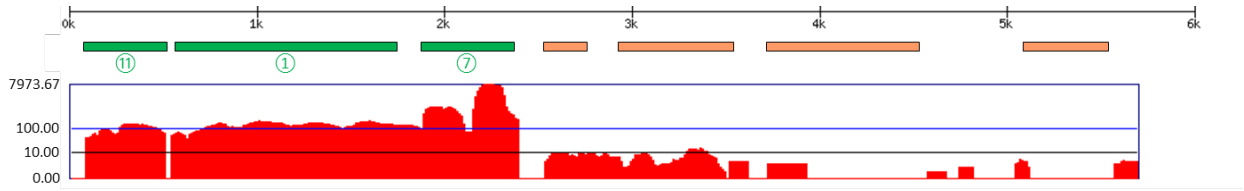
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

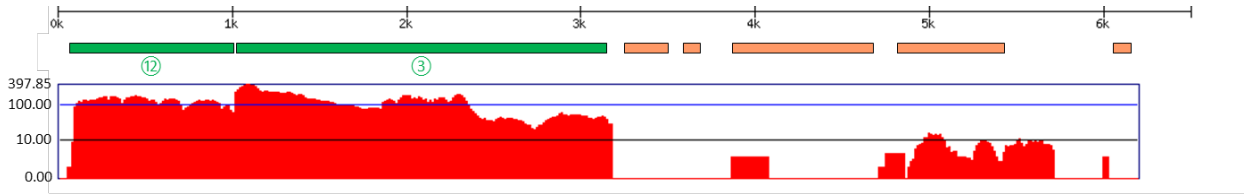
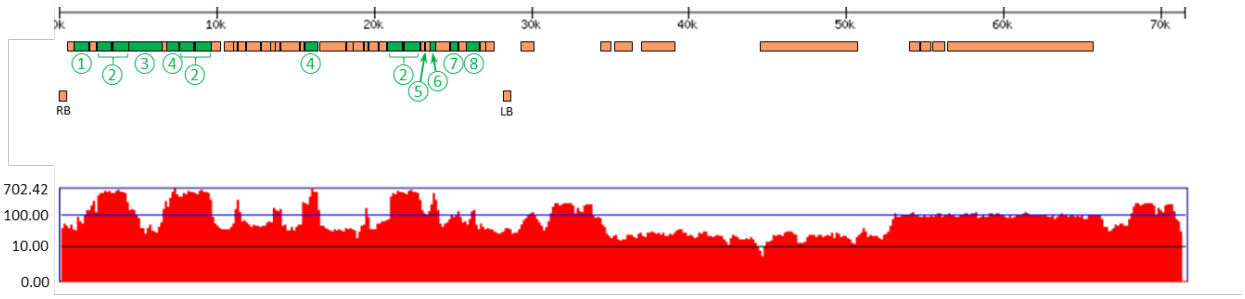


Figure 12. SbS Results for Control Maize

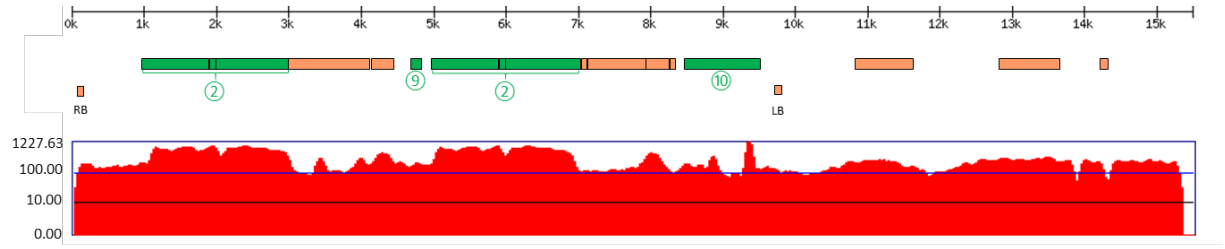
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome

(identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or plasmid backbone sequence present in the PHR03 control maize. **A)** SbS results for PHR03 control maize aligned against the intended insertion (16,176 bp; Figure 7). Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in the PHR03 control maize, and the sequence reads are solely due to the endogenous elements present in the PHR03 genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements.

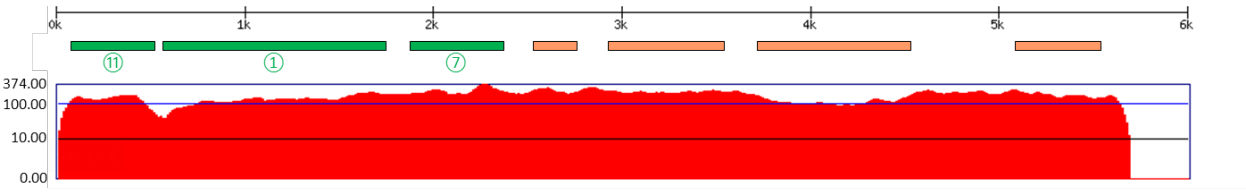
A. Alignment to PHP74643



B. Alignment to PHP56614



C. Alignment to PHP21139



D. Alignment to PHP31729

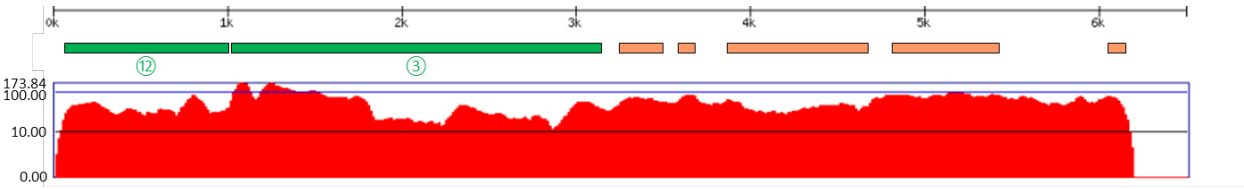
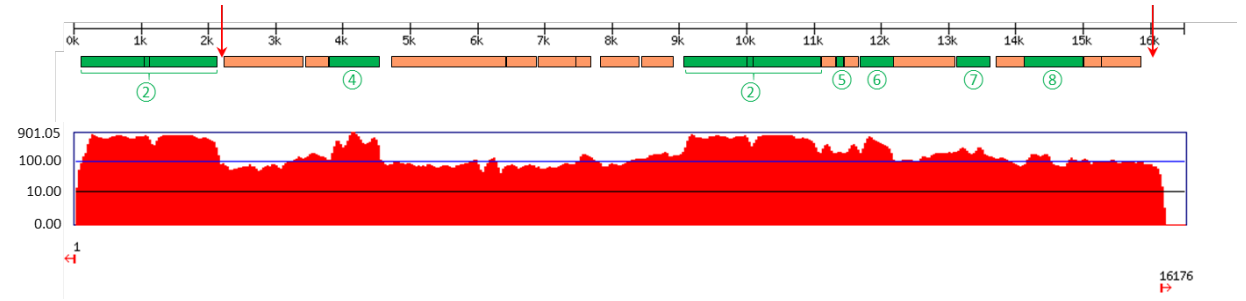


Figure 13. SbS Results for Positive Control Samples

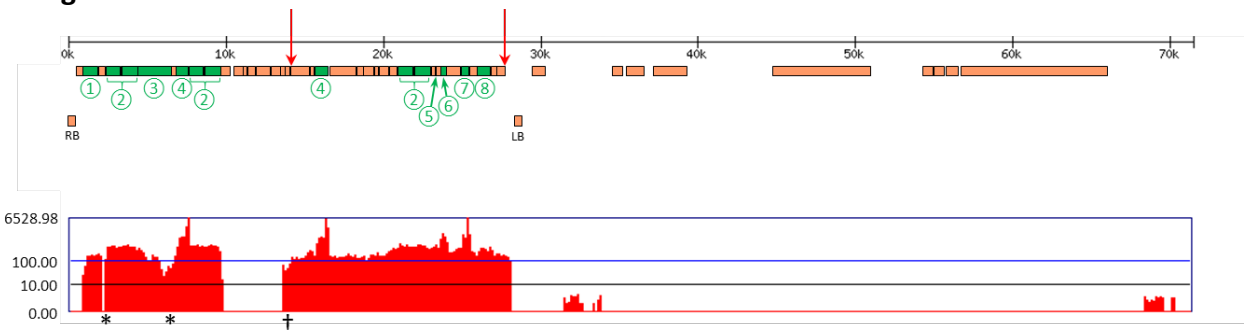
The positive control sample consisted of control maize DNA spiked with each plasmid at a level corresponding to one copy of plasmid per copy of the maize genome. The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in the plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. **A)** SbS results of the PHP74643 positive control sample aligned against PHP74643 (71,116 bp; Figure 5). Coverage was obtained across the full length of the plasmid, indicating successful capture of PHP74643 sequences by the SbS probe library. **B)** SbS results of the PHP56614 positive control sample aligned against PHP56614 (15,339 bp; Figure 1). Coverage was obtained across the full length of

the plasmid, indicating successful capture of PHP56614 sequences by the SbS probe library. **C)** SbS results of the PHP21139 positive control sample aligned against PHP21139 (5,687 bp; Figure 3). Coverage was obtained across the full length of the plasmid, indicating successful capture of PHP21139 sequences by the SbS probe library. **D)** SbS results of the PHP31729 positive control sample aligned against PHP31729 (6,181 bp; Figure 4). Coverage was obtained across the full length of the plasmid, indicating successful capture of PHP31729 sequences by the SbS probe library.

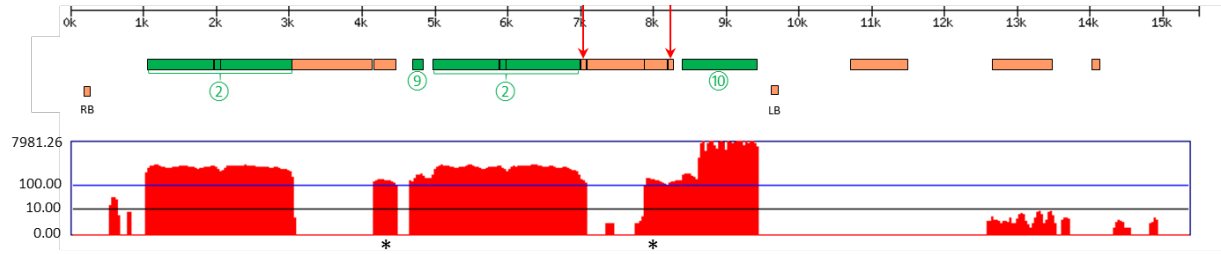
A. Alignment to Intended Insertion



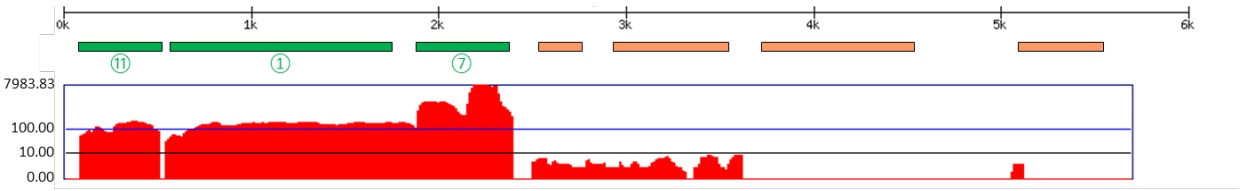
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

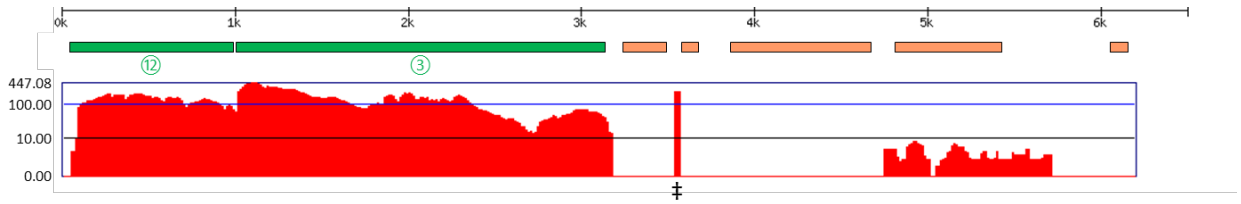


Figure 14. **SbS Results for a DP23211 Maize Plant Containing the Insertion (Plant ID 343210845)** The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant contains the intended insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the intended insertion (Figure 7). The presence of only two junctions demonstrates the presence of a single insertion in the DP23211 maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained for the elements between FRT1 and FRT87 transferred into DP23211 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region from approximately 1k to 10k that were not transferred into the DP23211 maize genome, and to the *pin*II terminator (*) and CaMV35S terminator (†) elements outside of the FRT sites due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in PHP74643. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained for *zm*-SEQ9, *zm*-SEQ8, the elements found in the intended insertion (between *zm*-SEQ9 to FRT1 and between FRT87 to *zm*-SEQ8), and for the endogenous elements not in the intended insertion (the *ubi*ZM1 promoter, 5' UTR, and intron in the I-*Cre*I cassette), along with the *pin*II terminator elements (*) in PHP56614 due to alignment of reads derived from the *pin*II terminator in the *pmi* cassette of the intended insertion to the two copies of this element in PHP56614. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained for the endogenous elements and for a small segment of an *att* recombination site that matches an *att* site found in the intended insertion (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or backbone sequence present in DP23211 maize.

V-C. Southern Blot Analysis

The DNA insertion in DP23211 maize was characterized by Southern blot analysis to evaluate the stability of the inserted *ipd072Aa*, *pmi*, and *mo-pat* gene cassettes and the DvSSJ1 fragment cassette.

All probes used for the analysis are indicated on the schematic maps of PHP74643 and the PHP74643 T-DNA region (Figure 15 and Figure 16, respectively) and outlined in Table 8.

Southern blot analysis was conducted on five generations of DP23211 maize to demonstrate the inserted DNA remained stable across multiple generations. Genomic DNA samples from individual plants of the T1, T2, T3, T4, and T5 generations of DP23211 maize and control maize were analyzed by digestion with restriction enzyme *Kpn* I and hybridization with the *pmi*, *mo-pat*, and *ipd072Aa* gene and DvSSJ1 fragment probes. The presence of equivalent bands from hybridization with the *pmi*, *mo-pat*, and *ipd072Aa* gene and DvSSJ1 fragment probes within all five generations analyzed confirms that the inserted DNA in DP23211 maize is stable and equivalent across multiple generations during the breeding process.

Kpn I was selected for use in Southern analysis because there is a single *Kpn* I restriction site within the DP23211 maize insertion (Figure 17), which provides a means to uniquely identify the event, as additional sites would be in the adjacent flanking genomic DNA. Genomic DNA samples from the five generations of DP23211 maize and control maize plants were digested with *Kpn* I and hybridized with the *pmi*, *mo-pat*, and *ipd072Aa* gene and DvSSJ1 fragment probes for Southern analysis. Hybridization patterns of these probes exhibited event-specific bands unique to the DP23211 maize insertion, and thus provided a means of verification that the genomic border region of the DP23211 maize insertion were not changed across the five generations during breeding. Plasmid PHP74643 was added to control maize DNA, digested with *Kpn* I, and included on the blot to verify successful probe hybridization.

Hybridization of the *pmi* and *mo-pat* probes to *Kpn* I-digested genomic DNA resulted in a consistent band of approximately 10,000 bp in all five generations of DP23211 maize (Table 9, Figure 18 and Figure 19, respectively). These results confirmed that the 5' border fragment, containing the *pmi* and *mo-pat* genes in the DP23211 maize insertion is intact and stable across the five generations of DP23211 maize. The plasmid lanes showed the expected band of 6,794 bp, confirming successful hybridization of the *pmi*, and *mo-pat* gene probes.

Hybridization of the DvSSJ1 fragment and the *ipd072Aa* gene probes to *Kpn* I-digested genomic DNA resulted in a single band of approximately 21,000 bp in all five generations of DP23211 maize samples analyzed (Table 9, Figure 20 and Figure 21, respectively). These results confirmed that the 3' border fragment, containing the DvSSJ1 fragment and *ipd072Aa* gene in the DP23211 maize insertion is intact and stable across the five generations of DP23211 maize. The plasmid

lanes showed the expected band of 32,601 bp, confirming successful hybridization of the DvSSJ1 fragment and *ipd072Aa* gene probes.

The Southern blot analysis with *DvSSJ1*, *ipd072Aa*, *pmi*, and *mo-pat* gene probes showed that the 5' and 3' genomic borders of the DP23211 insertion are intact and stable across five generations of DP23211 maize during the breeding process.

Materials and methods for Southern blot analysis of DP23211 maize are described in Appendix 3.

Table 8. Description of DNA Probes used for Southern Hybridization

Genetic Element/Probe Name	Probe Length (bp)	Position on PHP74643 T-DNA (bp to bp)[a]
pmi gene[b]	569	13,964 to 14,532
	660	14,502 to 15,161
mo-pat gene	582	18,611 to 19,192
DvSSJ1 fragment	236	22,789 to 23,024
ipd072Aa gene	264	26,712 to 26,975

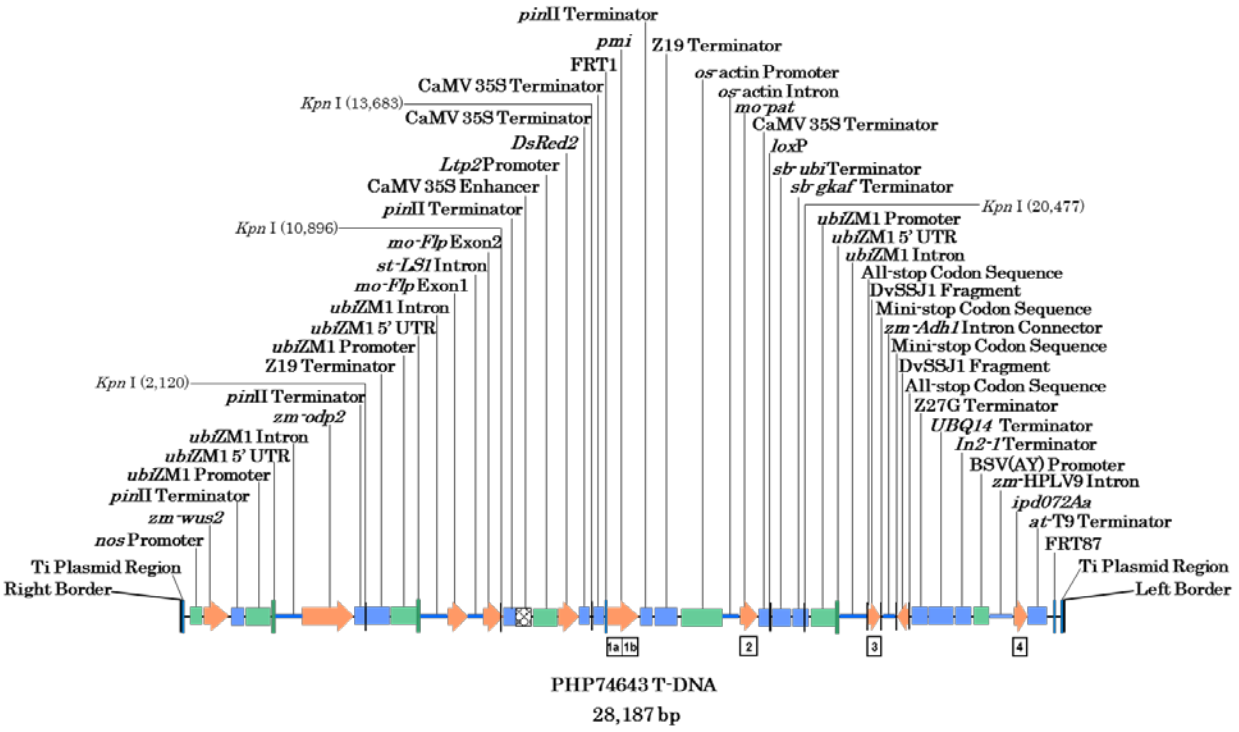
[a] The probe position is based on the PHP74643 T-DNA map (Figure 16)
[b] This probe comprises two fragments that are combined in a single hybridization solution

Table 9. Predicted and Observed Hybridization Bands on Southern Blots; *Kpn* I Digest

Probe Name	Predicted and Observed Fragment Size from Plasmid PHP74643 (bp)	Predicted Fragment Size from Intended Insertion Map of DP23211 Maize (bp)	Observed Fragment Size in DP23211 Maize[a] (bp)	Figure
pmi gene	6,794	>8,877	~10,000	18
mo-pat gene	6,794	>8,877	~10,000	19
DvSSJ1 fragment	32,601	>8,475	~21,000	20
ipd072Aa gene	32,601	>8,475	~21,000	21

[a] Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker III and VII fragments on the Southern blots. Due to inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.

Figure 15. **Schematic Diagram of Plasmid PHP74643 Indicating Restriction Enzyme Sites**
Schematic diagram of plasmid PHP74643 indicating *Kpn* I restriction enzyme sites with base pair positions. Right and Left Borders flank the T-DNA (Figure 16) that was transferred into the plant cell during transformation. The plasmid size is 71,116 bp.



Probe Number	Probe Name
1a and 1b	*pmi* gene
2	*mo-pat* gene
3	DvSSJ1 fragment
4	*ipd072Aa* gene

Figure 16. Schematic Diagram of T-DNA Region from Plasmid PHP74643 Indicating Restriction Enzymes and Southern Probe Locations

Schematic diagram of T-DNA region from plasmid PHP74643 indicating the *Kpn* I restriction enzyme sites, the *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes and the DvSSJ1 fragment cassette located between the FRT1 and FRT87 sites and intended for insertion into the landing pad, and the *zm-wus2*, *zm-odp2*, *mo-Flp*, and *DsRed2* gene cassettes located outside the FRT1 and FRT87 sites. The T-DNA region size is 28,187 bp. The portion of the T-DNA between the FRT1 and FRT87 sites is incorporated in the final DNA insertion (Figure 7). The locations of the Southern blot probes are shown by the boxes below the map.

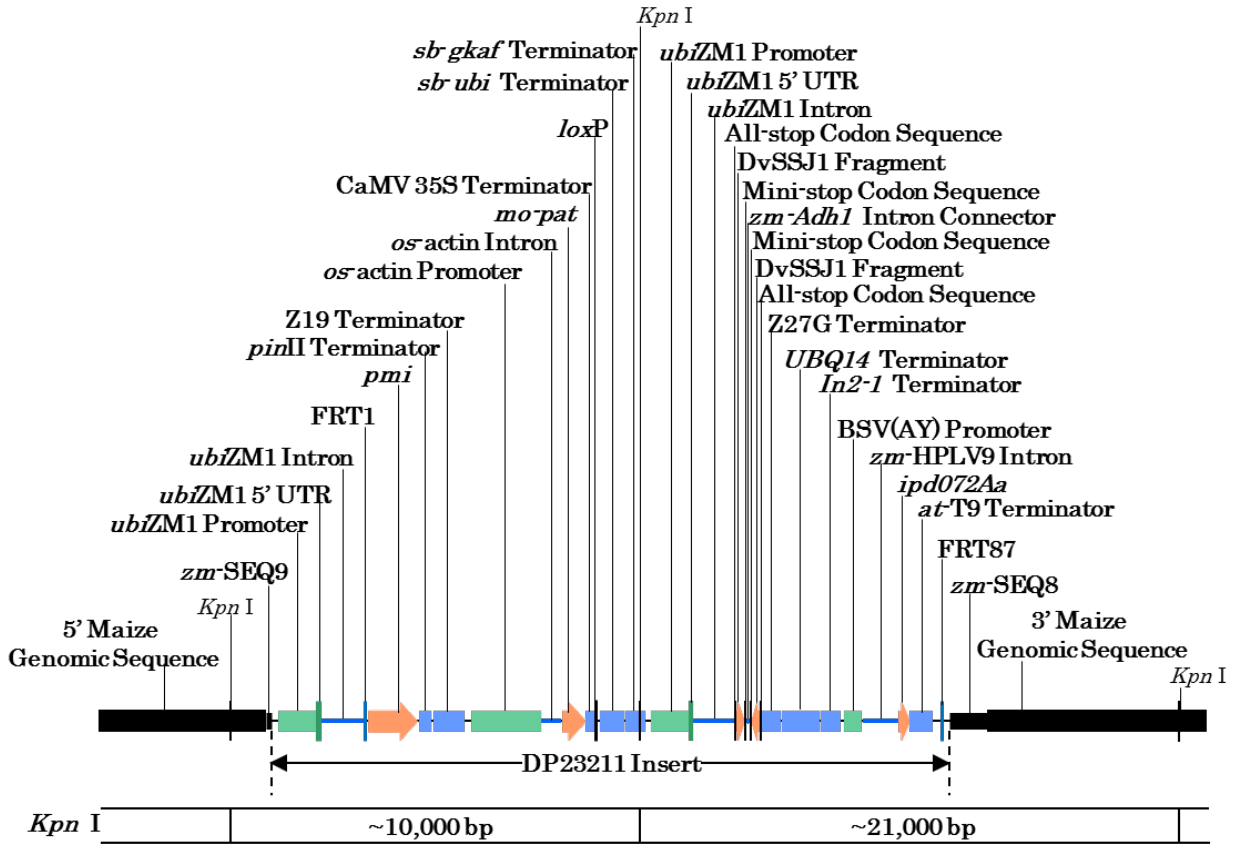
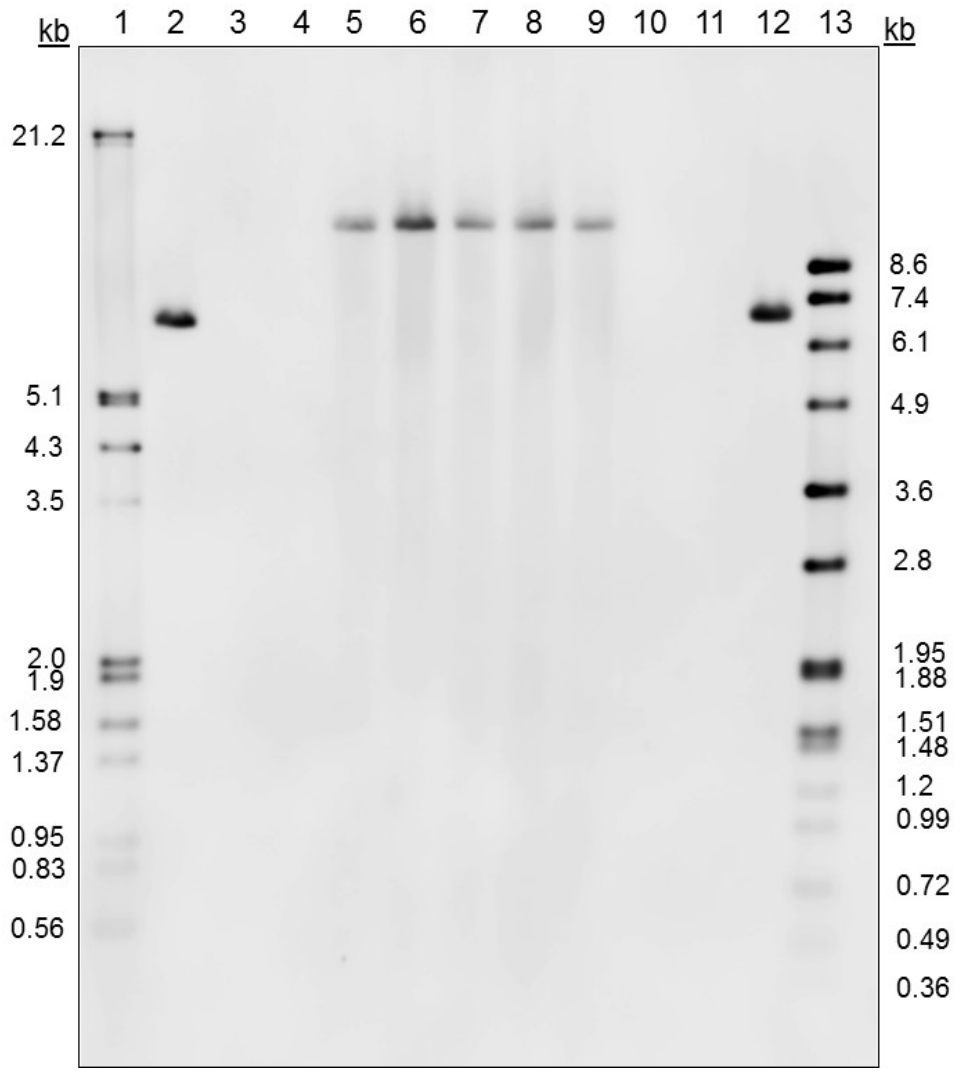


Figure 17. Map of the Final DNA Insertion in the DP23211 Maize Genome Following SSI Integration of the Gene Cassettes from the PHP74643 T-DNA

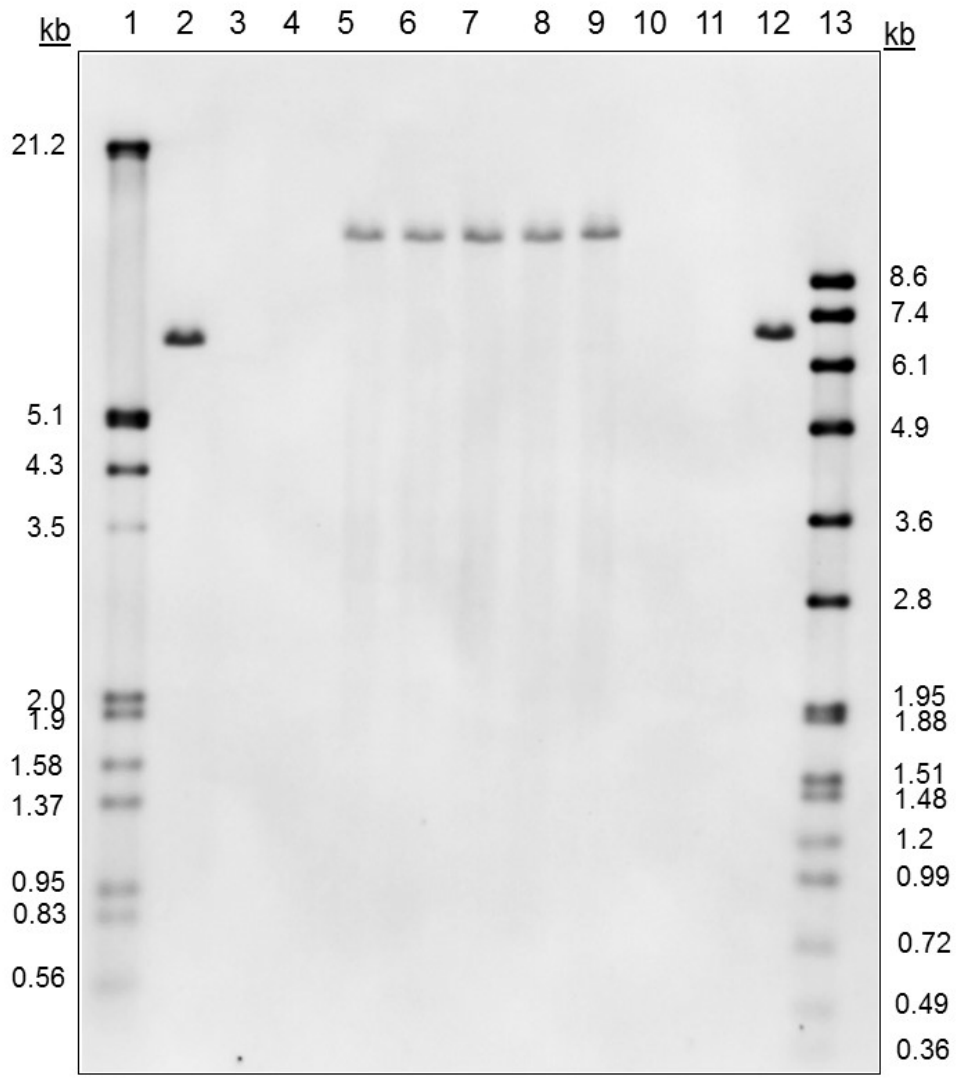
The DP23211 maize DNA insertion comprises sequences from two sources: the parts of the landing pad outside the FRT1 and FRT87 sites and the sequences from the PHP74643 plasmid surrounded by the FRT1 and FRT87 sites (with *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes and the DvSSJ1 fragment cassette). The flanking maize genomic DNA is represented by the horizontal black rectangular bars. *Kpn* I restriction sites are indicated with the sizes of the observed fragments on Southern blots shown below the map in base pairs (bp). The locations of restriction enzymes in the flanking maize genomic DNA are not to scale.



Lane	Sample	Lane	Sample
1	DIG-labeled DNA marker III	8	DP23211 maize T4 generation
2	1 copy of PHP74643 + PHR03 maize	9	DP23211 maize T5 generation
3	PHR03 control maize	10	Blank
4	Blank	11	PHR03 control maize
5	DP23211 maize T1 generation	12	1 copy of PHP74643 + PHR03 maize
6	DP23211 maize T2 generation	13	DIG-labeled DNA marker VII
7	DP23211 maize T3 generation		

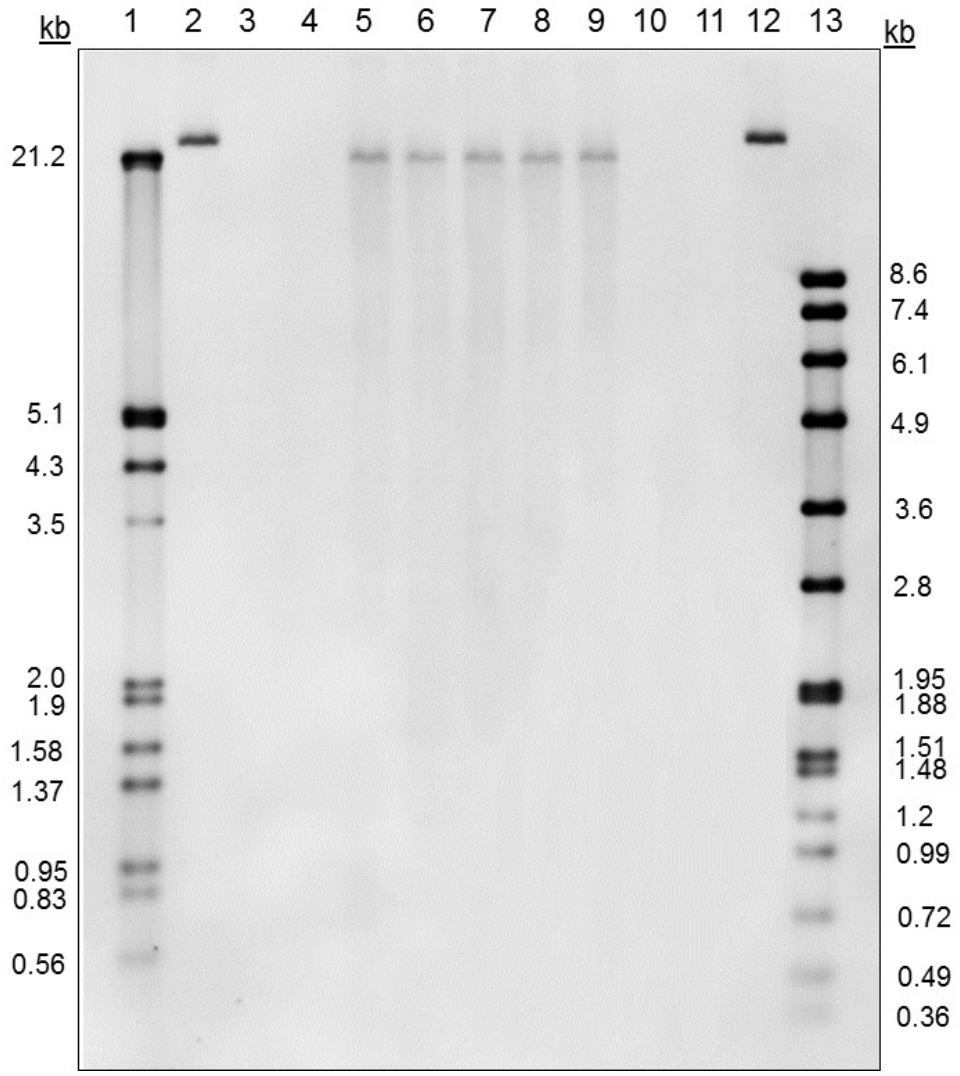
Figure 18. **Southern Blot Analysis of DP23211 Maize; *Kpn* I Digest with *pmi* Gene Probe**

Genomic DNA isolated from leaf tissues of DP23211 maize from the T1, T2, T3, T4, T5 generations and PHR03 control maize plants, was digested with *Kpn* I and hybridized to the *pmi* gene probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP74643 plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).



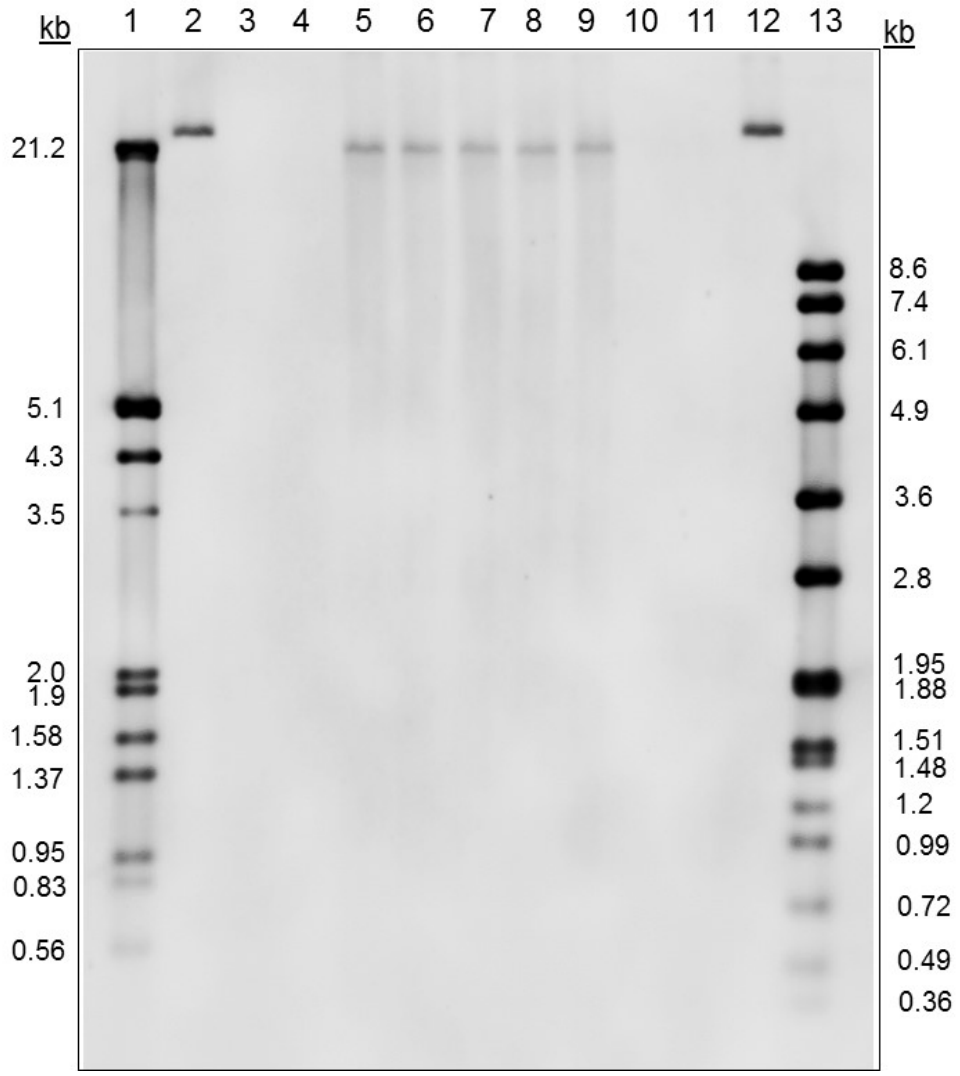
Lane	Sample	Lane	Sample
1	DIG-labeled DNA marker III	8	DP23211 maize T4 generation
2	1 copy of PHP74643 + PHR03 maize	9	DP23211 maize T5 generation
3	PHR03 control maize	10	Blank
4	Blank	11	PHR03 control maize
5	DP23211 maize T1 generation	12	1 copy of PHP74643 +PHR03 maize
6	DP23211 maize T2 generation	13	DIG-labeled DNA marker VII
7	DP23211 maize T3 generation		

Figure 19. **Southern Blot Analysis of DP23211 Maize; *Kpn* I Digest with *mo-pat* Gene Probe**
Genomic DNA isolated from leaf tissues of DP23211 maize from the T1, T2, T3, T4, T5 generations and PHR03 control maize plants, was digested with *Kpn* I and hybridized to the *mo-pat* gene probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP74643 plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).



Lane	Sample	Lane	Sample
1	DIG-labeled DNA marker III	8	DP23211 maize T4 generation
2	1 copy of PHP74643 + PHR03 maize	9	DP23211 maize T5 generation
3	PHR03 control maize	10	Blank
4	Blank	11	PHR03 control maize
5	DP23211 maize T1 generation	12	1 copy of PHP74643 + PHR03 maize
6	DP23211 maize T2 generation	13	DIG-labeled DNA marker VII
7	DP23211 maize T3 generation		

Figure 20. **Southern Blot Analysis of DP23211 Maize; *Kpn* I Digest with DvSSJ1 Fragment Probe**
Genomic DNA isolated from leaf tissues of DP23211 maize from the T1, T2, T3, T4, T5 generations and PHR03 control maize plants, was digested with *Kpn* I and hybridized to the DvSSJ1 fragment probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP74643 plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).



Lane	Sample	Lane	Sample
1	DIG-labeled DNA marker III	8	DP23211 maize T4 generation
2	1 copy of PHP74643 + PHR03 maize	9	DP23211 maize T5 generation
3	PHR03 control maize	10	Blank
4	Blank	11	PHR03 control maize
5	DP23211 maize T1 generation	12	1 copy of PHP74643 + PHR03 maize
6	DP23211 maize T2 generation	13	DIG-labeled DNA marker VII
7	DP23211 maize T3 generation		

Figure 21. **Southern Blot Analysis of DP23211 Maize; *Kpn* I Digest with *ipd072Aa* Gene Probe**
Genomic DNA isolated from leaf tissues of DP23211 maize from the T1, T2, T3, T4, T5 generations and PHR03 control maize plants, was digested with *Kpn* I and hybridized to the *ipd072Aa* gene probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP74643 plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).

V-D. Open Reading Frame Analysis

A bioinformatics assessment of translated Open Reading Frames (ORFs) of length ≥ 30 amino acids at the insertion site of DP23211 maize for similarity to known and putative allergens and toxins was conducted following established international criteria (Codex Alimentarius Commission, 2003; FAO/WHO, 2001a).

None of the putative translated ORFs at the DP23211 maize insertion site returned alignments from the search against the Comprehensive Protein Allergen Resource (COMPARE) 2019 database (January 2019 available at http://comparedatabase.org).

Safety evaluations of PMI have been previously disclosed by the developer (Syngenta Biotechnology, Inc.) (Vlachos and Huber, 2011; Ward and Huber, 2007). There was one region of sequence homology of eight contiguous identical amino acids between the identified translated ORF corresponding to the PMI protein and a known allergen, α-parvalbumin from Rana species CH2001 (unidentified edible frog) (Hilger et al., 2002). Further investigation using sensitive serum screening methodology (Codex Alimentarius Commission, 2003) demonstrated no cross-reactivity between the PMI protein and the serum from the single individual known to have demonstrated IgE-mediated allergy to this specific α-parvalbumin. The patient's serum did not recognize any portion of the PMI protein as an allergenic epitope. Therefore, the sequence identity between PMI protein and the α-parvalbumin from Rana species CH2001 is not biologically meaningful and has no implications for the potential allergenicity of PMI. No other putative translated ORFs at the DP23211 maize insertion site displayed any eight-contiguous amino acid exact matches to an allergen in the database.

These data indicate that it is unlikely that any of the identified translated ORFs at the DP23211 maize insertion site result in human or animal allergenicity.

None of the putative ORFs at the DP23211 maize insertion site returned alignments from the search against the Pioneer toxin database, indicating that it is unlikely that any of the putative ORFs at the DP23211 maize insertion site result in human or animal toxicity.

V-E. Inheritance and Genetic Stability of the Introduced Traits in DP23211 Maize

The stability of the inserted DNA during the breeding process is evaluated by examining the inheritance and segregation of the genes and/or traits in multiple generations. The segregation of these genes or traits as a single unit and as a single genetic locus will confirm that the inserted DNA will be predictably and stably inherited through the commercial breeding process.

Genotypic and phenotypic analyses were conducted for five generations of DP23211 maize (BC1F1 in genetic background PH1V5T, BC2F1, T1, T5, and BC1F1 in genetic background PH2SRH). The genotypic analysis evaluated each individual plant for the presence or absence of the

DP23211 insertion and the following genes or genetic elements by quantitative polymerase chain reaction (qPCR): *ipd072Aa, mo-pat, pmi, DvSSJ1,* and DP23211 event-specific PCR. The genotypic analysis also evaluated each individual plant for the presence or absence of the following genes or genetic elements by endpoint PCR: *STOPS2-UBI1, AT-T9-STOPS3, ATTB2-S2-BSV*. The phenotypic analysis evaluated tolerance to glufosinate-ammonium for each individual plant. The individual results for each plant were compared to the qPCR results to verify co-segregation of genotype with phenotype.

For the BC1F1, BC2F1, and T1 segregating generations of DP23211 maize, a chi-square test (at the 0.05 significance level) was conducted for qPCR results to compare the observed segregation ratios to the expected segregation ratio of 1:1. A chi-square test was not performed for the T5 generation of DP23211 maize as all plants were identified as positive (i.e., not segregating) as expected for a homozygous generation.

A summary of segregation results for DP23211 maize (BC1F1, BC2F1, and T1 generations) chi-square values and P-values is provided in Table 10. For each individual plant, all genotypic results (*i.e.*, PCR results) matched the corresponding phenotypic result (*i.e.,* herbicide tolerance result).

Genotypic and phenotypic results generated in this study demonstrated the inserted T-DNA and its included genetic elements within DP23211 maize segregated together and in accordance with Mendelian rules of inheritance for a single genetic locus. For each individual plant in all generations, the genotypic result was the same as the corresponding phenotypic result, indicating the DNA insertion co-segregated with the trait phenotype and was stable through traditional breeding.

Materials and methods for multi-generation segregation analysis of DP23211 maize are described in Appendix 4.

Table 10. **Summary of Segregation Results of Five Generations of DP23211 Maize**

Entry	Generation	Expected Segregation Ratio	Observed Segregation			Statistical Analysis	
		(Positive:Negative)	Positive	Negative	Total	Chi-Square[a]	P-Value
1	BC1F1 in genetic background PH1V5T	1:1	46	54	100	0.64	0.4237
2	BC2F1	1:1	50	50	100	0.00	1.0000
3	T1	1:1	52	48	100	0.16	0.6892
4	T5	Homozygous	100	0	100	--	--
5	BC1F1 in genetic background PH2SRH	1:1	50	50	100	0.00	1.0000

[a] Degrees of freedom = 1. A Chi-Square value greater than 3.84 (P-value less than 0.05) would indicate a significant difference.

V-F. Conclusions on the Molecular Characterization and Genetic Stability of DP23211 Maize

SbS and Southern blot analysis were conducted to characterize the DNA insertion in DP23211 maize. SbS analysis confirmed that a single, intact copy of the intended insertion was inserted into the maize genome and the intactness of the insertion was maintained. SbS analysis results also showed no plasmid backbone sequences or other unintended sequences were incorporated into DP23211 maize.

Southern blot analysis was conducted on five generations of DP23211 maize to demonstrate the inserted DNA remained stable across multiple generations. Genomic DNA samples from individual plants of the T1, T2, T3, T4, and T5 generations of DP23211 maize and control maize were analyzed by digestion with restriction enzyme *Kpn* I and hybridization with the *pmi*, *mo-pat*, *ipd072Aa*, and DvSSJ1 fragment probes. The presence of equivalent bands from hybridization with the *pmi*, *mo-pat*, *ipd072Aa*, and DvSSJ1 fragment probes within all five generations analyzed confirms that the inserted DNA in DP23211 maize is stable and equivalent across multiple generations during the breeding process.

Bioinformatic analysis supports the conclusion that there is no allergenicity concern regarding the putative translated ORFs at the DP23211 maize insertion site.

None of the putative translated ORFs at the DP23211 maize insertion site returned alignments from the search against the Pioneer toxin database, supports the conclusion that there is no toxicity concern regarding the putative translated ORFs at the DP23211 maize insertion site.

The inheritance and genetic stability of the inserted DNA was confirmed in 5 generations of DP23211 maize. The results of this analysis were consistent with the finding of a single locus of insertion in DP23211 maize that segregated according to Mendelian rules of inheritance. The stability of the insertion and of the herbicide tolerance phenotype was demonstrated in these populations.

Together, these analyses confirmed a single, intact, stable copy of the intended insertion, with no plasmid backbone or other unintended sequences, is present in the DP23211 genome, and there are no putative ORF allergen or toxin concerns in DP23211 maize.

VI. Compositional Assessment

An assessment of the compositional equivalence of a GE product compared to that of a conventional non-GE comparator with a history of safe use in food and feed is an important part of the weight-of-evidence approach used to evaluate the safety of genetically engineered plant products (Codex Alimentarius Commission, 2008; OECD, 1993). Compositional assessments of DP23211 maize were evaluated in comparison to concurrently grown non-GE, near-isoline maize (referred to as control maize) to identify statistical differences, and subsequently were evaluated in the context of normal ranges of variation established from multiple sources of non-GE, commercial maize data.

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected and analyzed for key nutritional components. The forage assessment included proximate, fiber, and mineral analytes. The grain assessment included proximate, fiber, fatty acid, amino acid, mineral, vitamin, secondary metabolite, and anti-nutrient analytes. Statistical analyses were conducted to evaluate and compare the nutrient composition of forage and grain derived from DP23211 maize and the control maize. If there was a statistical difference between DP23211 maize and the control maize for a given analyte, one or more reference ranges (*i.e.,* tolerance interval, literature range, and in-study reference range) representing the non-GE maize population with a history of safe use were utilized to evaluate statistical differences in the context of natural variation.

The analytes included for the compositional assessment were based on the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002).

VI-A. Generation of Tissue Samples for Nutrient Composition Analysis

The field portion of this study was conducted during the 2018 growing season at eight sites in commercial maize-growing regions of the United States (two sites in Illinois and one site in each of Iowa, Indiana, Minnesota, Pennsylvania, and Texas) and Canada (one site in Ontario). A randomized complete block design with four blocks was utilized at each site. Each block included DP23211 maize, non-genetically modified (non-GE) near-isoline control maize (referred to as control maize), and four of the following non-GE commercial maize lines: P0604, 2R602, 35A52, P0760, BK5883, XL5939, P0928, P0993, XL5828, BK6076, XL6158, P1105, P1151, and P1197 (referred to as reference maize).

VI-B. Determination of Nutrient Composition Analyte Concentrations

All procedures and methods for nutrient composition analyses of maize forage and grain were conducted in accordance with the requirements for the United States EPA Good Laboratory Practice (GLP) Standards, 40 CFR §160. The analytical procedures used were validated methods. The majority were based on methods published by AOAC International, AACC (American

Association of Cereal Chemists), and AOCS (American Oil Chemists' Society). Details regarding the methods used for nutrient composition analysis are provided in Appendix 10. Materials and Methods for Nutrient Composition Assessment.

VI-C. Assessment of Nutrient Composition Data

A total of 79 analytes were included in the statistical analysis, which included 78 original analytes, as well as one additional calculated analyte (total tocopherols, as described in Appendix 10). A total of 69 analytes (9 analytes from forage, and 60 analytes from grain) were evaluated using mixed model analysis. Three analytes did not meet criteria for sufficient quantities of observations above the LLOQ and were therefore subjected to Fisher's exact test. No statistical analysis was conducted on the remaining 7 analytes as all data values were below the LLOQ.

No statistically significant differences were observed between DP23211 maize and the control maize for 66 of the 79 analytes that went through across-site analysis via either mixed model analysis or Fisher's exact test. A statistically significant difference, before FDR adjustment, between DP23211 maize and the control maize grain was observed in the across-site analysis for oleic acid (C18:1), arachidic acid (C20:0), eicosenoic Acid (C20:1), vitamin B6 (pyridoxine), α-tocopherol, and p-coumaric acid in grain samples.

For the analytes where a statistical difference was identified prior to FDR adjustment, either all sample values for DP23211 maize were within the tolerance interval (arachidic acid (C20:0), α-tocopherol, eicosenoic acid (C20:1), oleic acid (C18:1), p-coumaric acid) or had some sample values above or below the tolerance interval limit but were within the literature range (vitamin B6 (pyridoxine). Those analytes with sample values within the tolerance interval and/or literature range indicate DP23211 maize is within the range of normal variation for these analytes and the statistical differences are not biologically meaningful.

Nutrient composition analysis results are provided in Table 118, Table 119, Table 120, Table 121, Table 122, Table 123, and Table 124 Details regarding statistical analysis methods are provided in Appendix 10.

VI-C.1. Proximates, Fiber, and Minerals in DP23211 Maize Forage

Proximates, fiber, and minerals were analyzed in forage derived from DP23211 maize and control maize. Results are shown in Table 11. No statistically significant differences (P-value < 0.05) were observed between DP23211 maize and control maize.

The results of the analysis of proximates, fiber (Acid Detergent Fiber (ADF) and Neutral Detergent Fiber (NDF)), and minerals in maize forage demonstrate that DP23211 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE commercial maize.

Table 11. Proximates, Fiber, and Mineral Results for DP23211 Maize Forage

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Proximates, Fiber, and Mineral Composition (% Dry Weight)						
Crude Protein	Mean	7.71	7.78	3.44 - 12.4	2.37 - 16.32	4.79 - 10.5
	Range	5.53 - 9.94	3.94 - 10.3			
	Confidence Interval	6.75 - 8.67	6.82 - 8.73			
	Adjusted P-Value	--	0.944			
	P-Value	--	0.725			
Crude Fat	Mean	4.14	3.93	0.784 - 6.17	ND - 6.755	2.42 - 6.27
	Range	2.88 - 6.02	2.73 - 5.38			
	Confidence Interval	3.66 - 4.62	3.45 - 4.41			
	Adjusted P-Value	--	0.833			
	P-Value	--	0.234			
Crude Fiber	Mean	25.0	25.4	14.1 - 30.8	12.5 - 42	13.9 - 32.2
	Range	17.6 - 32.5	18.4 - 33.9			
	Confidence Interval	22.7 - 27.3	23.1 - 27.7			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.585			
ADF	Mean	32.5	32.6	15.4 - 39.6	5.13 - 47.39	18.9 - 41.5
	Range	23.3 - 42.0	23.6 - 43.1			
	Confidence Interval	29.9 - 35.0	30.1 - 35.1			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.869			
NDF	Mean	50.2	51.3	28.1 - 63.7	18.30 - 67.80	29.7 - 61.5
	Range	42.0 - 64.3	41.9 - 60.7			
	Confidence Interval	47.3 - 53.3	48.4 - 54.4			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.366			
Ash	Mean	5.08	4.86	2.39 - 9.40	0.66 - 13.20	2.61 - 7.57
	Range	2.46 - 7.83	3.19 - 7.74			
	Confidence Interval	4.38 - 5.77	4.16 - 5.55			
	Adjusted P-Value	--	0.796			
	P-Value	--	0.208			
Carbohydrates	Mean	83.0	83.6	76.9 - 91.6	73.3 - 92.9	76.9 - 89.4
	Range	77.9 - 87.0	77.3 - 91.2			
	Confidence Interval	81.2 - 84.8	81.8 - 85.4			
	Adjusted P-Value	--	0.796			
	P-Value	--	0.206			
Calcium	Mean	0.273	0.249	0.0736 - 0.531	0.04 - 0.58	0.110 - 0.434
	Range	0.170 - 0.422	0.139 - 0.438			
	Confidence Interval	0.233 - 0.314	0.209 - 0.289			
	Adjusted P-Value	--	0.550			
	P-Value	--	0.0739			
Phosphorus	Mean	0.237	0.246	0.0844 - 0.428	0.07 - 0.55	0.159 - 0.371
	Range	0.114 - 0.361	0.144 - 0.375			
	Confidence Interval	0.203 - 0.272	0.211 - 0.281			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.516			

Note: Not detectable (ND): assay values that are below the lower limit of quantification (LLOQ) and cannot be quantified.

VI-C.2. Proximates and Fiber in DP23211 Maize Grain

Proximates and fiber were analyzed in grain derived from DP23211 maize and near-isoline control maize. Results are shown in Table 12. No statistically significant differences (P-value < 0.05) were observed between DP23211 maize and control maize.

The results of the analysis of proximates and fiber in maize grain demonstrate that DP23211 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE commercial maize.

Table 12. Proximates and Fiber Results for DP23211 Maize Grain

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Proximates and Fiber Composition (% Dry Weight)						
Total Dietary Fiber	Mean	9.71	9.43	3.15 – 21.8	5.78 - 35.31	4.44 - 13.4
	Range	8.19 - 11.7	7.67 - 12.0			
	Confidence Interval	9.18 - 10.2	8.89 - 9.96			
	Adjusted P-Value	--	0.833			
	P-Value	--	0.260			
Crude Protein	Mean	10.6	10.6	6.66 - 13.3	5.72 - 17.26	6.95 - 11.3
	Range	8.44 - 12.0	7.94 - 11.7			
	Confidence Interval	9.98 - 11.3	9.98 - 11.3			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.999			
Crude Fat	Mean	4.25	4.22	2.34 - 5.90	1.363 - 7.830	3.66 - 5.41
	Range	3.63 - 4.87	3.48 - 4.94			
	Confidence Interval	4.09 - 4.42	4.05 - 4.39			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.495			
Crude Fiber	Mean	2.43	2.50	1.57 - 3.61	0.49 - 5.5	2.02 - 3.14
	Range	1.97 - 2.71	2.13 - 3.02			
	Confidence Interval	2.28 - 2.58	2.35 - 2.65			
	Adjusted P-Value	--	0.667			
	P-Value	--	0.155			
ADF	Mean	4.22	4.27	2.64 - 6.24	1.41 - 11.34	3.20 - 5.69
	Range	3.22 - 4.84	3.04 - 5.16			
	Confidence Interval	3.92 - 4.53	3.96 - 4.57			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.637			
NDF	Mean	10.5	10.4	7.49 - 18.6	4.28 - 24.3	8.15 - 13.3
	Range	8.12 - 12.8	8.18 - 13.4			
	Confidence Interval	9.73 - 11.3	9.60 - 11.2			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.526			
Ash	Mean	1.37	1.40	1.01 - 1.87	0.616 - 6.282	1.04 - 1.49
	Range	1.24 - 1.49	1.24 - 1.54			
	Confidence Interval	1.33 - 1.41	1.36 - 1.44			
	Adjusted P-Value	--	0.667			
	P-Value	--	0.150			
Carbohydrates	Mean	83.7	83.7	80.5 - 88.5	77.4 - 89.7	82.9 - 87.6
	Range	82.4 - 85.9	82.6 - 86.1			
	Confidence Interval	83.1 - 84.4	83.1 - 84.3			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.932			

VI-C.3. Fatty Acids in DP23211 Maize Grain

Fatty acids were analyzed in grain derived from DP23211 maize and near-isoline control maize. Results are shown in Table 13.

A statistically significant difference (P-value < 0.05), prior to FDR adjustment, was observed between DP23211 maize and control maize mean values for oleic acid (C18:1), arachidic acid (C20:0), and eicosenoic acid (C20:1); however, all the individual values were within the tolerance interval, indicating DP23211 maize is within the range of normal variation for these fatty acids and the statistical differences are not biologically meaningful. The non-significant FDR-adjusted P-values indicate that these differences were likely false positives.

The results of the analysis of fatty acids in maize grain demonstrate that DP23211 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE commercial maize.

Table 13. Fatty Acids Results for DP23211 Maize Grain

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Fatty Acid Composition (% Total Fatty Acids)						
Lauric Acid (C12:0)	Mean	0.101	0.0899	0 - 0.209[b]	ND - 0.698	0.0360 - 0.271
	Range	0.0439 - 0.300	0.0432 - 0.311			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Myristic Acid (C14:0)	Mean	<LLOQ[a]	<LLOQ[a]	0 - 0.267[b]	ND - 0.288	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Palmitic Acid (C16:0)	Mean	13.5	13.5	9.33 - 24.7	6.81 - 39.0	11.1 - 18.0
	Range	13.1 - 13.9	13.0 - 14.0			
	Confidence Interval	13.3 - 13.7	13.3 - 13.6			
	Adjusted P-Value	--	0.985			
	P-Value	--	0.800			
Palmitoleic Acid (C16:1)	Mean	0.118	0.115	0 - 0.445	ND - 0.67	0.0562 - 0.195
	Range	0.0543 - 0.127	0.0514 - 0.149			
	Confidence Interval	0.114 - 0.122	0.111 - 0.119			
	Adjusted P-Value	--	0.833			
	P-Value	--	0.251			
Heptadecanoic Acid (C17:0)	Mean	0.0734	0.0822	0 - 0.236	ND - 0.203	0.0382 - 0.141
	Range	0.0454 - 0.105	0.0459 - 0.117			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Heptadecenoic Acid (C17:1)	Mean	<LLOQ[a]	<LLOQ[a]	0 - 0.135[b]	ND - 0.131	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Stearic Acid (C18:0)	Mean	1.73	1.76	1.31 - 3.83	ND - 4.9	1.60 - 2.33
	Range	1.58 - 1.94	1.60 - 2.02			
	Confidence Interval	1.64 - 1.82	1.68 - 1.85			
	Adjusted P-Value	--	0.550			
	P-Value	--	0.0583			
Oleic Acid (C18:1)	Mean	21.5	21.1	17.3 - 38.6	16.38 - 42.81	20.0 - 32.8
	Range	20.7 - 22.3	20.2 - 22.1			
	Confidence Interval	21.1 - 21.8	20.8 - 21.5			
	Adjusted P-Value	--	0.264			
	P-Value	--	0.00765*			
Linoleic Acid (C18:2)	Mean	60.1	60.4	30.7 - 65.5	13.1 - 67.68	49.8 - 62.6
	Range	58.6 - 60.8	59.0 - 61.5			
	Confidence Interval	59.7 - 60.4	60.0 - 60.7			
	Adjusted P-Value	--	0.550			
	P-Value	--	0.0698			
α-Linolenic Acid (C18:3)	Mean	1.70	1.70	0 - 1.90	ND - 2.33	1.35 - 2.02
	Range	1.54 - 1.84	1.47 - 1.94			
	Confidence Interval	1.64 - 1.76	1.65 - 1.76			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.962			

Table 13. Fatty Acids Results for DP23211 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Fatty Acid Composition (% Total Fatty Acids)						
Arachidic Acid (C20:0)	Mean	0.361	0.367	0.295 - 0.872	0.267 - 1.2	0.328 - 0.539
	Range	0.332 - 0.399	0.337 - 0.399			
	Confidence Interval	0.348 - 0.373	0.355 - 0.379			
	Adjusted P-Value	--	0.454			
	P-Value	--	0.0395*			
Eicosenoic Acid (C20:1)	Mean	0.306	0.313	0 - 0.614	ND - 1.952	0.233 - 0.425
	Range	0.266 - 0.334	0.290 - 0.331			
	Confidence Interval	0.300 - 0.311	0.308 - 0.319			
	Adjusted P-Value	--	0.377			
	P-Value	--	0.0245*			
Eicosadienoic Acid (C20:2)	Mean	<LLOQ[a]	0.0519	0 - 0.825[b]	ND - 2.551	0.0339 - 0.185
	Range	<LLOQ[a]	0.0407 - 0.110			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Behenic Acid (C22:0)	Mean	0.191	0.178	0 - 0.423	ND - 0.5	0.100 - 0.298
	Range	0.0951 - 0.227	0.0912 - 0.247			
	Confidence Interval	0.172 - 0.206	0.157 - 0.195			
	Adjusted P-Value	--	0.658			
	P-Value	--	0.115			
Lignoceric Acid (C24:0)	Mean	0.278	0.279	0 - 0.639	ND - 0.91	0.252 - 0.501
	Range	0.244 - 0.311	0.250 - 0.354			
	Confidence Interval	0.269 - 0.287	0.270 - 0.289			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.646			

Note: Fatty acids analyte erucic acid (C22:1) was not statistically analyzed because all sample values in the current study and in historical commercial reference lines were below the lower limit of quantification (LLOQ). This analyte was excluded from the report table. Not applicable (NA); mixed model analysis was not performed or confidence interval was not determined. Not detectable (ND): one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified.

[a] < LLOQ, all fatty acid sample values were below the assay LLOQ.

[b] Historical reference data range was provided as tolerance interval was not calculated since the data did not meet the assumptions of any tolerance interval calculation method.

* A statistically significant difference (P-Value < 0.05) was observed.

VI-C.4. Amino Acids in DP23211 Maize Grain

Amino acids were analyzed in grain derived from DP23211 maize and near-isoline control maize. Results are shown in Table 14. No statistically significant differences (P-value < 0.05) were observed between DP23211 maize and control maize.

The results of the analysis of amino acids in maize grain demonstrate that DP23211 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE commercial maize.

Table 14. Amino Acids Results for DP23211 Maize Grain

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Amino Acid Composition (% Dry Weight)						
Alanine	Mean	0.812	0.818	0.457 - 1.07	0.40 - 1.48	0.505 - 0.889
	Range	0.601 - 0.929	0.565 - 0.929			
	Confidence Interval	0.755 - 0.869	0.761 - 0.875			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.644			
Arginine	Mean	0.455	0.453	0.302 - 0.598	0.12 - 0.71	0.356 - 0.486
	Range	0.388 - 0.500	0.378 - 0.500			
	Confidence Interval	0.433 - 0.477	0.431 - 0.475			
	Adjusted P-Value	--	0.944			
	P-Value	--	0.722			
Aspartic Acid	Mean	0.683	0.689	0.414 - 0.901	0.30 - 1.21	0.475 - 0.730
	Range	0.538 - 0.756	0.522 - 0.764			
	Confidence Interval	0.643 - 0.723	0.649 - 0.729			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.430			
Cystine	Mean	0.220	0.224	0.132 - 0.295	0.12 - 0.51	0.114 - 0.276
	Range	0.160 - 0.288	0.110 - 0.301			
	Confidence Interval	0.197 - 0.244	0.200 - 0.248			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.499			
Glutamic Acid	Mean	2.12	2.14	1.11 - 2.76	0.83 - 3.54	1.25 - 2.32
	Range	1.55 - 2.45	1.45 - 2.43			
	Confidence Interval	1.96 - 2.28	1.98 - 2.30			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.600			
Glycine	Mean	0.391	0.389	0.285 - 0.485	0.184 - 0.685	0.304 - 0.423
	Range	0.345 - 0.423	0.317 - 0.428			
	Confidence Interval	0.373 - 0.410	0.370 - 0.407			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.647			
Histidine	Mean	0.317	0.315	0.190 - 0.380	0.14 - 0.46	0.202 - 0.325
	Range	0.270 - 0.360	0.238 - 0.351			
	Confidence Interval	0.299 - 0.335	0.297 - 0.333			
	Adjusted P-Value	--	0.944			
	P-Value	--	0.714			
Isoleucine	Mean	0.376	0.382	0.213 - 0.498	0.18 - 0.69	0.235 - 0.404
	Range	0.287 - 0.428	0.271 - 0.432			
	Confidence Interval	0.351 - 0.402	0.356 - 0.407			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.458			
Leucine	Mean	1.41	1.43	0.694 - 1.85	0.60 - 2.49	0.759 - 1.53
	Range	1.03 - 1.67	0.923 - 1.63			
	Confidence Interval	1.30 - 1.52	1.32 - 1.53			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.599			
Lysine	Mean	0.309	0.306	0.178 - 0.396	0.129 - 0.668	0.254 - 0.346
	Range	0.258 - 0.352	0.276 - 0.354			
	Confidence Interval	0.296 - 0.321	0.293 - 0.318			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.420			

Table 14. Amino Acids Results for DP23211 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Amino Acid Composition (% Dry Weight)						
Methionine	Mean	0.206	0.213	0.120 - 0.328	0.10 - 0.47	0.0934 - 0.268
	Range	0.163 - 0.253	0.110 - 0.264			
	Confidence Interval	0.181 - 0.231	0.188 - 0.238			
	Adjusted P-Value	--	0.658			
	P-Value	--	0.107			
Phenylalanine	Mean	0.572	0.570	0.303 - 0.736	0.24 - 0.93	0.318 - 0.590
	Range	0.449 - 0.674	0.374 - 0.648			
	Confidence Interval	0.528 - 0.615	0.526 - 0.614			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.917			
Proline	Mean	1.04	1.04	0.557 - 1.26	0.46 - 1.75	0.641 - 1.07
	Range	0.804 - 1.19	0.735 - 1.16			
	Confidence Interval	0.972 - 1.11	0.972 - 1.11			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.984			
Serine	Mean	0.540	0.545	0.307 - 0.685	0.15 - 0.91	0.348 - 0.572
	Range	0.434 - 0.614	0.395 - 0.603			
	Confidence Interval	0.508 - 0.573	0.512 - 0.577			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.592			
Threonine	Mean	0.393	0.396	0.245 - 0.491	0.17 - 0.67	0.270 - 0.410
	Range	0.331 - 0.434	0.305 - 0.427			
	Confidence Interval	0.373 - 0.412	0.376 - 0.415			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.546			
Tryptophan	Mean	0.0650	0.0668	0.0376 - 0.0991	0.027 - 0.215	0.0512 - 0.0843
	Range	0.0490 - 0.0791	0.0528 - 0.0877			
	Confidence Interval	0.0612 - 0.0688	0.0630 - 0.0706			
	Adjusted P-Value	--	0.658			
	P-Value	--	0.134			
Tyrosine	Mean	0.312	0.311	0.170 - 0.557	0.10 - 0.73	0.192 - 0.359
	Range	0.252 - 0.396	0.214 - 0.367			
	Confidence Interval	0.287 - 0.337	0.286 - 0.336			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.891			
Valine	Mean	0.482	0.489	0.307 - 0.629	0.21 - 0.86	0.329 - 0.513
	Range	0.386 - 0.536	0.367 - 0.542			
	Confidence Interval	0.454 - 0.511	0.460 - 0.517			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.390			

VI-C.5. Minerals in DP23211 Maize Grain

Minerals were analyzed in grain derived from DP23211 maize and near-isoline control maize. Results are shown in Table 15. No statistically significant differences (P-value < 0.05) were observed between DP23211 maize and control maize.

The results of the analysis of minerals in maize grain demonstrate that DP23211 maize is comparable to is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE commercial maize.

Table 15. Minerals Results for DP23211 Maize Grain

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Mineral Composition (% Dry Weight)						
Calcium	Mean	0.00361	0.00346	0.00167 - 0.00872	ND - 0.101	0.00215 - 0.00650
	Range	0.00245 - 0.00538	0.00231 - 0.00496			
	Confidence Interval	0.00316 - 0.00406	0.00301 - 0.00391			
	Adjusted P-Value	--	0.550			
	P-Value	--	0.0798			
Copper	Mean	0.0000904	0.0000903	<0.0000625[a] - 0.000411	ND - 0.0021	<0.0000625[a] - 0.000194
	Range	<0.0000625[a] - 0.000134	<0.0000625[a] - 0.000130			
	Confidence Interval	0.0000684 - 0.000112	0.0000683 - 0.000112			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.981			
Iron	Mean	0.00176	0.00176	0.00123 - 0.00308	0.0000712 - 0.0191	0.000955 - 0.00245
	Range	0.00139 - 0.00222	0.00121 - 0.00239			
	Confidence Interval	0.00157 - 0.00194	0.00157 - 0.00195			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.958			
Magnesium	Mean	0.132	0.132	0.0809 - 0.159	0.0035 - 1.000	0.0858 - 0.133
	Range	0.117 - 0.142	0.117 - 0.148			
	Confidence Interval	0.127 - 0.136	0.127 - 0.136			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.843			
Manganese	Mean	0.000747	0.000740	0.000327 - 0.00123	0.0000312 - 0.0054	0.000359 - 0.000870
	Range	0.000466 - 0.00101	0.000361 - 0.000979			
	Confidence Interval	0.000625 - 0.000868	0.000619 - 0.000861			
	Adjusted P-Value	--	0.914			
	P-Value	--	0.663			
Phosphorus	Mean	0.359	0.357	0.207 - 0.415	0.010 - 0.750	0.264 - 0.373
	Range	0.331 - 0.403	0.325 - 0.400			
	Confidence Interval	0.350 - 0.368	0.349 - 0.366			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.617			
Potassium	Mean	0.354	0.355	0.255 - 0.534	0.020 - 0.720	0.306 - 0.486
	Range	0.311 - 0.441	0.313 - 0.429			
	Confidence Interval	0.339 - 0.368	0.341 - 0.369			
	Adjusted P-Value	--	0.963			
	P-Value	--	0.754			
Sodium	Mean	0.000491	0.000414	<LLOQ[a] - 0.0151	ND - 0.150	<0.0000625[a] - 0.00953
	Range	<0.0000625[a] - 0.00596	<0.0000625[a] - 0.00518			
	Confidence Interval	0.000217 - 0.00111	0.000183 - 0.000938			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.622			
Zinc	Mean	0.00208	0.00208	0.00140 - 0.00347	0.0000283 - 0.0043	0.00132 - 0.00312
	Range	0.00152 - 0.00264	0.00154 - 0.00266			
	Confidence Interval	0.00187 - 0.00229	0.00187 - 0.00229			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.956			

Not detectable (ND): one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified.

[a] < LLOQ, one or more sample values were below the assay LLOQ.

[b] Historical reference data range was provided as tolerance interval was not calculated since the data did not meet the assumptions of any tolerance interval calculation method.

VI-C.6. Vitamins in DP23211 Maize Grain

Vitamins were analyzed in grain derived from DP23211 maize and near-isoline control maize. Results are shown inTable 16.

A statistically significant difference (P-value < 0.05), before FDR adjustment, was observed between DP23211 maize and control maize mean values for vitamin B6 (pyridoxine) and α-tocopherol. For α-tocopherol, all individual values were within the tolerance interval. Some of the individual values for vitamin B6 (pyridoxine) were above or below the tolerance interval limit, but were within the literature range. These results indicate that DP23211 maize is within the range of normal variation for these vitamins and the statistical differences are not biologically meaningful. The non-significant FDR-adjusted P-values indicate that these differences were likely false positives.

The results of the analysis of vitamins in maize grain demonstrate that DP23211 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE commercial maize.

Table 16. Vitamins Results for DP23211 Maize Grain

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Vitamin Composition (mg/kg Dry Weight)						
β-Carotene	Mean	0.283	0.302	0.0330 - 4.24	0.3 - 5.4	0.0996 - 1.71
	Range	0.111 - 0.475	0.171 - 0.531			
	Confidence Interval	0.209 - 0.357	0.228 - 0.376			
	Adjusted P-Value	--	0.658			
	P-Value	--	0.126			
Vitamin B1 (Thiamine)	Mean	2.43	2.42	1.74 - 5.38	ND - 40.00	1.58 - 2.91
	Range	1.85 - 2.80	1.95 - 2.91			
	Confidence Interval	2.33 - 2.53	2.32 - 2.52			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.835			
Vitamin B2 (Riboflavin)	Mean	<0.900[a]	<0.900[a]	<0.900[a] - 2.27[b]	ND - 7.35	<0.900[a]
	Range	<0.900[a]	<0.900[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Vitamin B3 (Niacin)	Mean	13.0	13.1	7.85 - 32.5	ND - 70	9.29 - 18.0
	Range	10.9 - 17.9	10.6 - 17.7			
	Confidence Interval	12.2 - 13.7	12.3 - 13.9			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.555			
Vitamin B5 (Pantothenic Acid)	Mean	5.46	5.50	2.42 - 7.53	3.01 - 14	4.56 - 6.95
	Range	3.46 - 6.57	4.56 - 6.22			
	Confidence Interval	5.09 - 5.83	5.13 - 5.87			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.318			
Vitamin B6 (Pyridoxine)	Mean	2.99	2.76	1.61 - 8.88	ND - 12.14	1.62 - 5.26
	Range	2.00 - 4.65	1.40 - 4.09			
	Confidence Interval	2.63 - 3.35	2.40 - 3.12			
	Adjusted P-Value	--	0.377			
	P-Value	--	0.0273[*]			
Vitamin B9 (Folic Acid)	Mean	1.17	1.27	0.323 - 2.44	ND - 3.50	0.280 - 3.63
	Range	0.400 - 2.20	0.322 - 2.08			
	Confidence Interval	0.939 - 1.41	1.04 - 1.51			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.526			
α-Tocopherol	Mean	3.37	3.00	0 - 23.5	ND - 68.67	<0.500[a] - 19.3
	Range	<0.500[a] - 7.22	<0.500[a] - 7.39			
	Confidence Interval	1.80 - 4.93	1.43 - 4.56			
	Adjusted P-Value	--	0.377			
	P-Value	--	0.0243			
β-Tocopherol	Mean	<0.500[a]	<0.500[a]	<0.500[a] - 1.10[b]	ND - 19.80	<0.500[a] - 0.863
	Range	<0.500[a]	<0.500[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
γ-Tocopherol	Mean	10.8	10.6	0 - 44.8	ND - 58.61	2.19 - 31.2
	Range	<1.00[a] - 17.8	<1.00[a] - 19.5			
	Confidence Interval	7.08 - 14.5	6.92 - 14.3			
	Adjusted P-Value	--	0.964			
	P-Value	--	0.769			

Table 16. Vitamins Results for DP23211 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Vitamin Composition (mg/kg Dry Weight)						
δ-Tocopherol	Mean	<0.500[a]	<0.500[a]			
	Range	<0.500[a]	<0.500[a]			
	Confidence Interval	NA	NA	<0.500[a] - 2.61[b]	ND - 14.61	<0.500[a] - 1.68
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Total Tocopherols	Mean	14.6	14.1			
	Range	<2.50[a] - 24.3	<2.50[a] - 26.3			
	Confidence Interval	9.47 - 19.8	8.95 - 19.3	0 - 59.1	ND - 89.91	4.56 - 40.7
	Adjusted P-Value	--	0.911			
	P-Value	--	0.425			

Note: Not applicable (NA); mixed model analysis was not performed or confidence interval was not determined. Not detectable (ND): one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified.

[a] < LLOQ, one or more sample values were below the assay LLOQ.

[b] Historical reference data range was provided as tolerance interval was not calculated since the data did not meet the assumptions of any tolerance interval calculation method.

* A statistically significant difference (P-Value < 0.05) was observed.

VI-C.7. Secondary Metabolites and Anti-Nutrients in DP23211 Maize Grain

Secondary metabolites and anti-nutrients were analyzed in grain derived from DP23211 maize and near-isoline control maize. Results are shown inTable 17.

A statistically significant difference (P-value < 0.05), before FDR adjustment, was observed between DP23211 maize and control maize mean values for *p*-coumaric acid; however, all the individual values were within the tolerance interval, indicating DP23211 maize is within the range of normal variation for *p*-coumaric acid and the statistical difference is not biologically meaningful. The non-significant FDR-adjusted P-value indicates that this difference was likely a false positive.

The results of the analysis of secondary metabolites and anti-nutrients in maize grain demonstrate that DP23211 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE commercial maize.

Table 17. Secondary Metabolites and Anti-Nutrients Results for DP23211 Maize Grain

Analyte	Reported Statistics	Control Maize	DP23211 Maize	Tolerance Interval	Literature Range	Reference Data Range
Secondary Metabolite and Anti-Nutrient Composition (% Dry Weight or as Indicated)						
p-Coumaric Acid	Mean	0.0218	0.0198	0.00742 - 0.0492	ND - 0.08	0.0132 - 0.0403
	Range	0.0161 - 0.0298	0.0159 - 0.0294			
	Confidence Interval	0.0199 - 0.0236	0.0179 - 0.0217			
	Adjusted P-Value	--	0.168			
	P-Value	--	0.00244*			
Ferulic Acid	Mean	0.233	0.236	0.123 - 0.349	0.02 - 0.44	0.164 - 0.298
	Range	0.185 - 0.284	0.199 - 0.306			
	Confidence Interval	0.219 - 0.247	0.222 - 0.250			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.491			
Furfural	Mean	<0.000100[a]	<0.000100[a]	<0.0000500[a]	ND	<0.000100[a]
	Range	<0.000100[a]	<0.000100[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Inositol	Mean	0.0257	0.0264	0.00966 - 0.0548	0.00613 - 0.257	0.0157 - 0.0450
	Range	0.0180 - 0.0433	0.0181 - 0.0371			
	Confidence Interval	0.0224 - 0.0291	0.0230 - 0.0297			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.312			
Phytic Acid	Mean	1.08	1.08	0.493 - 1.33	ND - 1.940	0.696 - 1.21
	Range	0.891 - 1.34	0.853 - 1.38			
	Confidence Interval	1.02 - 1.14	1.02 - 1.14			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.931			
Raffinose	Mean	0.135	0.126	0 - 0.396	ND - 0.466	<0.0800[a] - 0.339
	Range	<0.0800[a] - 0.264	<0.0800[a] - 0.239			
	Confidence Interval	0.0847 - 0.186	0.0755 - 0.177			
	Adjusted P-Value	--	0.833			
	P-Value	--	0.266			
Trypsin Inhibitor (TIU/mg DW)	Mean	2.50	2.54	1.03 - 9.18	ND - 8.42	1.64 - 3.21
	Range	2.16 - 3.23	1.98 - 3.38			
	Confidence Interval	2.28 - 2.73	2.31 - 2.76			
	Adjusted P-Value	--	0.911			
	P-Value	--	0.341			

Note: Not applicable (NA); mixed model analysis was not performed or confidence interval was not determined. Not detectable (ND): one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified. Trypsin inhibitor units per milligram dry weight (TIU/mg DW).

[a] < LLOQ, one or more sample values were below the assay LLOQ.

* A statistically significant difference (P-Value < 0.05) was observed.

VI-D. Conclusions on Compositional Assessment of DP23211 Maize

A compositional comparative assessment was conducted to determine if DP23211 maize grain and forage are compositionally equivalent to conventional maize varieties represented by non-GE near-isoline control maize and non-GE commercial maize.

The compositional analyses of grain included crude protein, crude fat, crude fiber, ADF, NDF, total dietary fiber, ash, carbohydrates, fatty acids, amino acids, vitamins, minerals, key anti-nutrients, and key secondary metabolites. Compositional analyses of forage included crude protein, crude fat, crude fiber, ADF, NDF, ash, carbohydrates, calcium, and phosphorus. In total, data from 69 different analytical components (60 in grain, nine in forage), and one calculated component, were presented and discussed.

No statistically significant differences were observed between DP23211 maize and the control maize for 66 of the 79 analytes that went through across-site analysis via either mixed model analysis or Fisher's exact test. A statistically significant difference, before FDR adjustment, between DP23211 maize and the control maize grain was observed in the across-site analysis for oleic acid (C18:1), arachidic acid (C20:0), eicosenoic acid (C20:1), vitamin B6 (pyridoxine), α-tocopherol, and *p*-coumaric acid.

For the analytes where a statistical difference was identified prior to FDR adjustment, either all sample values for DP23211 maize were within the tolerance interval (arachidic acid (C20:0), α-tocopherol, eicosenoic acid (C20:1), oleic acid (C18:1), *p*-coumaric acid) or had some sample values above or below the tolerance interval limit but were within the literature range (vitamin B6 (pyridoxine)). Those analytes with sample values within the tolerance interval and/or literature range indicate DP23211 maize is within the range of normal variation for these analytes and the statistical differences are not biologically meaningful.

Based on these analyses, the grain and forage of DP23211 maize are comparable to conventional maize with respect to nutrient composition and there would be no anticipated impact on raw or processed maize commodities.

VII. Characterization, Concentration, and Safety Assessment of the dsRNA and Proteins Expressed in DP23211 Maize

VII-A. Characterization of the Human and Livestock Safety of DvSSJ1 dsRNA in DP23211 Maize

The DvSSJ1 dsRNA produced in DP23211 maize is targeted to match a portion of the smooth septate junction protein 1 (*dvssj1*) gene sequence from WCR to down-regulate expression of the DvSSJ1 protein in the mid-gut of WCR via RNA interference (RNAi). DP23211 maize produces DvSSJ1 dsRNA which, when ingested by WCR, results in suppression of the DvSSJ1 protein in the intestinal lining. Reduction in DvSSJ1 protein expression and subsequent loss of formation of the gut epithelium barrier and cellular deformities are lethal to WCR (Hu et al., 2019).

In this section, the characterization and concentration of DvSSJ1 dsRNA is described and this information demonstrates that DP23211 maize is unlikely to present a hazard to human or animal health. The use of dsRNA is not novel, and RNA interference (RNAi) occurs regularly in nature. The concentration levels of DvSSJ1 dsRNA were measured to evaluate human and livestock exposure. Exposure to DvSSJ1 dsRNA through consumption of DP23211 maize is low for humans and livestock, and mammalian barriers exist that are anticipated to prevent or significantly reduce human and animal exposure to DvSSJ1 dsRNA from consumption of foods or feed containing DP23211 maize. Double-stranded RNA has a history of safe use, and consumption of DvSSJ1 dsRNA in food or feed containing DP23211 maize is not expected to present a hazard to human or animal health, therefore supporting the overall safety assessment of DP23211 maize.

VII-A.1. Double-stranded RNA (dsRNA) Interference

RNA interference (RNAi) is a naturally occurring mechanism for down-regulation of gene expression in most plants and animals, and it involves a process that promotes an RNA transcript's degradation when dsRNA is endogenously transcribed or exogenously introduced into a cell with a sequence that is complementary to the mRNA produced by transcription of a gene. There are many reviews that describe the RNAi machinery and provide a detailed mechanism of action of RNAi (for example, Fire et al., 1998; Kurreck, 2009; Mello and Conte, 2004; Price and Gatehouse, 2008). Several GM crops have, or are being developed using RNAi to improve taste or nutritional profile, provide resistance to viruses, and to control of insect pests (for example, Anderson *et al.*, 2016; Baum *et al.*, 2007; Bonfim *et al.*, 2007; Krieger *et al.*, 2008; Mao *et al.*, 2011; Pavely *et al.*, 2007). Therefore, the use of RNAi in the field of agricultural biotechnology has a history of safety, and the established ERA framework for GM crops is robust and suitable for assessing plants developed using RNAi (CERA, 2011a). The DvSSJ1 trait produces dsRNA, which down-regulates expression of the DvSSJ1 protein in the mid-gut of the WCR. The DvSSJ1 dsRNA has been shown to be highly specific, with activity limited to species within the family Chrysomelidae and the genus *Diabrotica* (see section VI.A.4. below)

VII-A.2. DvSSJ1 dsRNA Trait Expression Assessment

The concentration levels of DvSSJ1 dsRNA were evaluated in DP23211 maize. Tissue samples were collected during the 2018 growing season at six sites in commercial maize-growing regions of the United States and Canada. Site locations were selected to represent geographically diverse characteristics within the crop production areas, including different soil textures and weather variables. The six sites selected for field evaluation of DP23211 maize represented ideal growing regions suitable for the Comparative Relative Maturity (CRM) of DP23211 maize line (107 CRM). The remaining sites represented appropriate maize growing regions suitable for the CRM of DP23211 maize but were sites that provided a greater diversity of climatological and agronomic conditions in which to assess DP23211 maize (and DvSSJ1 dsRNA concentrations within various plant tissue types). Physical location map and further description of field site selection is detailed in Section VIII.

A randomized complete block design with four blocks was utilized at each site. The following tissue samples were collected: root (V6, V9, R1, R4, and R6 growth stages), leaf (V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), forage (R4 growth stage), whole plant (R1 and R6 growth stages), and grain (R6 growth stage). Concentrations of DvSSJ1 dsRNA were determined using a QuantiGene Plex Assay. Due to QuantiGene Plex assay specifications, fresh tissue was used for analysis of DvSSJ1 dsRNA expression. Expression values from fresh tissue were converted to dry weight expression values to provide consistency in evaluation of exposure to protein values presented later in this Petition for IPD072, PAT, and PMI, and to redcuce variation in measured values due to tissue moisture differences. Dry weight expression values were used to assess exposure of non-target organisms and insects beneficial to agriculture. DvSSJ1 dsRNA fresh tissue values as well as converted dry weight expression values are presented in Table 18.

Individual sample results below the LLOQ were assigned a value equal to half of the LLOQ for calculation purposes.

Table 18. Across-Site Summary of DvSSJ1 dsRNA Concentrations in DP23211 Maize

Tissue	ng DvSSJ1 dsRNA/mg Tissue Fresh Weight				ng DvSSJ1 ds RNA/mg Tissue Dry Weight		
	Growth Stage	Mean	Range	Standard Deviation	Mean	Range	Standard Deviation
Root	V6	0.00591	0.00373 – 0.00877	0.00146	0.0513	0.0222 – 0.0944	0.0208
	V9	0.00451	0.00244 – 0.00705	0.00127	0.0374	0.0195 – 0.0870	0.0148
	R1	0.00408	0.00242 – 0.00628	0.00107	0.0291	0.0152 – 0.0585	0.0100
	R4	0.00288	0.00134 – 0.00451	0.000836	0.0184	0.00777 – 0.0356	0.00669
	R6	0.00184	0.000190 – 0.00476	0.00134	0.0115	0.00150 – 0.0357	0.00831
Leaf	V9	0.0133	0.00659 – 0.0290	0.00484	0.0592	0.0343 – 0.0985	0.0134
	R1	0.0130	0.00561 – 0.0284	0.00513	0.0497	0.0235 – 0.0967	0.0179
	R4	0.0225	0.00980 – 0.0379	0.00695	0.0646	0.0243 – 0.113	0.0232
	R6	0.00810	0.00177 – 0.0267	0.00664	0.0132	0.00240 – 0.0331	0.0105
Pollen	R1	0.000559	0.000330 – 0.000960	0.000127	0.000987	0.000561 – 0.00202	0.000309
Forage	R4	0.00515	0.00264 – 0.0177	0.00331	0.0190	0.00977 – 0.0565	0.0107
Whole Plant	R1	0.00364	0.00223 – 0.00641	0.000924	0.0219	0.0127 – 0.0359	0.00510
	R6	0.00413	0.00153 – 0.0120	0.00245	0.0108	0.00459 – 0.0299	0.00540
Grain	R6	0.00322	0.00102 – 0.00727	0.00161	0.00413[a]	0.00122 – 0.0109	0.00236

Note: Growth stages (Abendroth et al., 2011).

[a] One sample from the respective tissue/growth stage was not included in the dry weight mean because fresh weight : dry weight ratio could not be calculated due to insufficient sample weight after lyophilization.

Additional details regarding analytical methods and calculations for trait expression analysis are provided in Appendix 6. Methods for Determination of DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI Protein Concentrations.

VII-A.3. Safety Assessment of dsRNA

Plants engineered to contain RNAi technology have been evaluated using the same global regulatory framework as other genetically modified plants, most commonly those that express novel transgenic protein(s). The regulatory framework was developed based on recommendations and guidelines from scientific and regulatory authorities (Chassy *et al.*, 2004; Codex Alimentarius Commission, 2008; EFSA Panel on Genetically Modified Organisms (GMO), 2011; FAO/WHO, 1996; FAO/WHO, 2000; FAO/WHO, 2001b; Jonas *et al.*, 1996; OECD, 1993; US-FDA, 1992; WHO, 1995). Safety assessment of GE plants is designed to evaluate the impact of intended effects of the genetic modification, as well as the impact of unintended effects that may result from the transformation process or the activity of the introduced trait. This assessment is science-based, comparative in nature, relies principally on the comparison of response variables in the GE crop with those of a conventional, non-GE counterpart with a history of safe use, and typically includes components such as agronomic evaluation, compositional assessment and molecular characterization. A tiered approach is applied for safety assessment of expressed trait products, which includes evaluations of history of safe use, mode of action and specificity, bioinformatics analyses, expression and intake assessments, and other measures of the potential for systemic exposure. In cases where specific hazards are identified that cannot otherwise be mitigated, specific toxicology studies may be conducted to inform the safety assessment. Application of the comparative assessment paradigm for evaluation of novel GE plants has proven effective over time, as commercialized GE crops have a well-established history of safe use and have not been associated with adverse effects in humans or animals based on their consumption as food and feed (Delaney et al., 2018; Flachowsky and Reuter, 2017; Sánchez and Parrott, 2017; Van Eenennaam and Young, 2014).

Recent investigations have considered the applicability of the current safety assessment framework to the evaluation of plants containing one or more dsRNA traits. The conclusions of regulatory authorities, academic scientists and industry professionals support that the existing regulatory framework as appropriate for plants containing RNAi technology, except that bioinformatics assessments for similarity to protein toxins or known allergens are not relevant due to the absence of novel protein expression (FSANZ, 2013; Parrott et al., 2010; Petrick et al., 2013; Sherman et al., 2015). Therefore, this historically accepted and robust approach was applied to the safety assessment of DvSSJ1 dsRNA expressed in DP23211 maize.

VII-A.3.a. History of Safe Consumption of RNA

The ubiquity and conservation of nucleic acids in the molecular biology of virtually all living organisms ensures that nearly every bite of food consumed by humans and animals contains RNA and DNA. Nucleic acids have always been present in human and animal food and feed, and their consumption has not been associated with adverse health effects (FSANZ, 2013; US-EPA, 2001b; US-FDA, 1992). RNA-mediated gene silencing (e.g. RNAi) is similarly conserved across eukaryotic

species, including plants, fungi and animals (Pickford and Cogoni, 2003). Reports of the detection of endogenous RNAi in plants and animals, including those used as food and feed, are plentiful in the peer-reviewed literature (Ambros, 2004; Della Vedova *et al.*, 2005; Frizzi and Huang, 2010; Hou *et al.*, 2017; Kusaba, 2004; Senda *et al.*, 2012; Tuteja *et al.*, 2004; Wagner *et al.*, 2015). These reports demonstrate a history of safe consumption of dsRNA in food and feed. Some investigators have identified RNA sequences, including long dsRNA in commonly consumed plants (Ivashuta et al., 2009; Jensen et al., 2013) and animals (Dever et al., 2015) with sequence complementarity to human and other animal genes, and without evidence of biologic activity or adverse effects, further supporting their history of safe consumption.

Knowledge and experience with the specificity and selectivity of RNAi has increased its utility in the development of agricultural biotechnology, with applications for insect control (Baum et al., 2007; Baum and Roberts, 2014), viral and fungal pathogen resistance (McLoughlin et al., 2018; Pence et al., 2016), nutritional alterations (Buhr et al., 2002; Stoutjesdijk et al., 2002; Tran et al., 2017), and alterations to improve processing and shelf-life stability or delay ripening (Krieger et al., 2008; Sheehy et al., 1988; Waltz, 2015). According to the International Service for the Acquisition of Agri-Biotech Applications, RNAi-based events have been approved in many jurisdictions for food, feed, or cultivation in multiple crops including: alfalfa, apple, bean, maize, papaya, potato, plum, soybean, squash, and tomato (ISAAA, 2019).

VII-A.3.b. Mammalian Barriers to Exposure to DvSSJ1 dsRNA Consumed in Food and Feed

The physical, chemical, enzymatic and molecular barriers to exposure and activity of dietary dsRNAs ingested by humans and other mammals have been well-described in the context of agricultural biotechnology and the safety assessment of crops containing RNAi technology (FSANZ, 2013; Sherman *et al.*, 2015; US-EPA, 2016). In the absence of identified and well-characterized mammalian transporters, the gut epithelium and vascular endothelium form a physical barrier to the uptake of hydrophilic macromolecules like dsRNAs and siRNAs. The low pH in the stomach and nucleases present in saliva and the lumen of the GI tract degrade free RNAs, thus reducing the potential for systemic exposure. Any dsRNA that could potentially cross the gut lumen and enter the systemic circulation would encounter additional nucleases in blood, resulting in further degradation, and be rapidly cleared via glomerular filtration in the kidneys. Further, for any small amount of dsRNA (such as DvSSJ1 dsRNA) that could potentially persist in systemic circulation to exert a biological effect, it would need to cross additional hydrophobic cellular membranes to penetrate cells of tissue parenchyma, escape endosomal capture and lysosomal degradation, accumulate in cytosol at sufficient cellular concentrations to impact gene regulation, and have a molecular target with which to interact (Sherman *et al.*, 2015).

The function of these well-defined barriers is exemplified by published information resulting from pharmaceutical industry efforts to improve systemic exposure to oligonucleotide-based

therapeutics via the oral route (Forbes and Peppas, 2012; Lorenzer *et al.*, 2015; O'Neill *et al.*, 2011). Current efforts to increase stability and target-site trafficking of oligonucleotide-based biotherapeutics include: chemical modifications (Geary *et al.*, 2015; Shukla *et al.*, 2010); macromolecular complexes, carriers, and conjugates (Loretz *et al.*, 2006; Moroz *et al.*, 2016a); encapsulation (Moroz *et al.*, 2016b); association with functional nanoparticles (Liu *et al.*, 2019; Rabanel *et al.*, 2012); and the use of permeability enhancers (Sánchez-Navarro *et al.*, 2016), often in combination. In consideration of potential oral exposure to dsRNA, it was recently demonstrated that repeated oral administration of dsRNA, or a pool of 21-nt siRNAs, specifically targeted to the murine ortholog of the vacuolar ATPase (*vATPase*) did not result in toxicologically-relevant or adverse effects in mice at high dose levels of 64 mg/kg BW/day (dsRNA) and 48 mg/kg BW/day (siRNA pool) (Petrick *et al.*, 2015). Additionally, gene expression analysis did not reveal evidence of vATPase gene suppression in tissues from the gastrointestinal tract, liver, kidneys, brain or bone. These described barriers to exposure and activity of ingested dsRNAs are anticipated to prevent or significantly reduce human and animal exposure to DvSSJ1 dsRNA from consumption of foods or feed containing DP23211 maize.

VII-A.3.b.1. Potential for Systemic Exposure to Ingested DvSSJ1 dsRNA in Vertebrates

The presence of dsRNA in conventional crops and vegetables commonly consumed in food and feed has been described (Jensen *et al.*, 2013). The DvSSJ1 dsRNA expressed in DP23211 maize is not anticipated to behave differently when ingested by humans or other animals. However, the potential for transfer and activity of ingested dsRNA or other small RNAs to humans and animals from food and feed has resulted in several published reports describing the detection of exogenous sequences in various body fluids (Chen *et al.*, 2016; Han and Luan, 2015; Link *et al.*, 2019). Some investigators have also reported functional impacts on gene expression (Baier *et al.*, 2014; Mlotshwa *et al.*, 2015; Wang *et al.*, 2017) occasionally associated with differences in health-based outcomes in animal models (Zhang *et al.*, 2012). Other investigators have reported contradictory results that do not support significant dietary exposure to small RNAs from food or feed (Micó *et al.*, 2016; Snow *et al.*, 2013; Witwer *et al.*, 2013). In some cases, data purporting to demonstrate cross-kingdom transfer and functional activity of small RNAs were not reproducible (Auerbach *et al.*, 2016; Dickinson *et al.*, 2013). Several reports of cross-kingdom transfer and biologic activity were recently reviewed during a United States Environmental Protection Agency (EPA) Scientific Advisory Panel (SAP) evaluating scientific issues impacting the human health and ecological risk assessments of plant-incorporated protectants (PIPs) containing RNAi technology (US-EPA, 2016). The panel concluded that the studies describing cross-kingdom transfer contained a number of experimental insufficiencies including: lack of appropriate controls, absence of sufficient data to support health-based conclusions, extrapolation of low-abundance targets detected *in vivo* to experimental effects of high-concentration exposures observed *in vitro* (Witwer and Halushka, 2016), and failure to consider stoichiometric estimates of target abundance and strength of siRNA-target interactions in the

facilitation of canonical regulatory functions (Snow *et al.*, 2013). Additionally, the Panel noted that improvements in analytical methods are required to address identified concerns with sensitivity and specificity related to the potential for environmental contamination or the presence of artifacts in biological samples (Lusk, 2014; Witwer, 2015; Witwer and Halushka, 2016). Questions about the validation of analytical methods (sensitivity, specificity and reproducibility) for reliable detection and quantification of small RNAs continue to challenge investigators, and have been highlighted as a significant hurdle which must be overcome (Chan and Snow, 2017; Kang *et al.*, 2017; Witwer and Halushka, 2016; Witwer and Zhang, 2017). Despite these methodologic and analytical challenges, and irrespective of the previously described arthropod-specific nature of the gene target and specificity of activity limited to the *Diabrotica* species within the Chrysomelidae family, the potential for systemic exposure of humans and animals to DvSSJ1 dsRNA from ingestion of foods and feed containing DP23211 maize cannot be definitively excluded; therefore, an assessment of human and livestock dietary exposure was conducted (see section VI-F. Human and Livestock Exposure to DvSSJ1 dsRNA, and the IPD072Aa, PAT, and PMI Proteins in DP23211 Maize).

VII-A.3.c. Human Exposure to DvSSJ1 dsRNA in DP23211 Maize

The intended use(s) and degree of exposure can be considered in assessing the safety of a GE crop. This consideration includes the effect(s) (if any) of the level of the food or food product in the diet, patterns of dietary consumption, and the defining characteristics of populations that consume the crop of interest (OECD, 1993).

The levels of expression of the DvSSJ1 dsRNA was measured in edible tissues and is reported in Table 18. These values were then used to estimate potential exposure to humans as stated below.

VII.A.3.c.1. DvSSJ1 dsRNA Human Exposure Assessment Methods

Dietary exposures to DvSSJ1 dsRNA were calculated utilizing the mean concentrations of DvSSJ1 dsRNA in DP23211 maize grain (presented in section VI-A.2. DvSSJ1 dsRNA Trait Expression Assessment), along with consumption data from the Dietary Exposure Evaluation Model – Food Commodity Intake Database (DEEM™ - FCID), Version 4.02 (DEEM/FCID, 2018; US-EPA, 2014). This model is commonly used by the U.S. EPA Office of Prevention, Pesticides and Toxic Substances to estimate human dietary exposure.

The DEEM™ - FCID model is designed to perform mean annual (chronic) and 95th percentile daily (acute) exposure analyses for the U.S. population and a wide range of sub-populations based on 2-day food consumption data from the National Health and Nutrition Examination Survey (NHANES; CDC, 2019) What We Eat in America (WWEIA) 2005-2010 (USDA-NAL, 2019). The 'foods-as-eaten' data in NHANES were converted to raw agricultural commodities and other basic ingredients based on the EPA/USDA FCID recipe set as of August 2014 (DEEM/FCID, 2018).

While the consumption and recipe data that form the basis of the DEEM™ - FCID model are derived from a U.S. survey, the data are applicable to other populations with similar dietary consumption patterns such as Canada, New Zealand, Japan and Italy (WHO-GEMS, 2019). In addition, dietary consumption patterns for specific sub-populations, for example Hispanics or Asians, are likely similar in the U.S. and other countries.

VII.A.3.c.2. DvSSJ1 dsRNA Human Exposure Assessment Results

Conservative total replacement scenarios were utilized for both acute and chronic exposures, assuming that maize in each foodstuff was derived from DP23211 maize grain. It was assumed that no degradation of proteins or dsRNA occurred during processing or cooking of corn flour, corn-flour-baby food, corn meal, corn meal-baby food and corn bran foodstuff categories. However, in the case of corn oil, corn starch, and corn syrup foodstuff categories, protein contents are considered to be zero due to processing (CRA, 2006a; CRA, 2006b; CRA, 2006c; Hefle and Taylor, 1999); therefore, consumption of these foodstuffs was not considered for exposure estimates.

Mean annual (chronic) exposures of DvSSJ1 dsRNA were highest for 'Hispanic', 'children ages 1-6 years', 'children ages 7-12 years', 'children ages 1-2 years', 'children ages 3-5 years', and 'children ages 6-12 years' subgroups at 0.000002 mg/kg BW/day.

The highest 95th percentile per capita daily (acute) exposures for DvSSJ1 dsRNA were in the 'children ages 1-6 years', 'children ages 1-2 years', and 'children ages 3-5 years' subgroups with an exposure of 0.000009 mg/kg BW/day for each group. The highest 95th percentile users daily (acute) exposures for DvSSJ1 dsRNA were in the 'children ages 1-2 years' and 'children ages 3-5 years' subgroups with an exposure of 0.000012 mg/kg BW/day for each group.

The actual exposure to DvSSJ1 dsRNA from DP23211 maize grain in the diet is expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus grain containing DvSSJ1 dsRNA will be mixed with other grain potentially not containing DvSSJ1 dsRNA, and (2) reductions in concentrations will likely occur during processing to produce maize flour and other processed commodities.

The estimated chronic DvSSJ1 dsRNA exposure was compared to the Threshold of Toxicological Concern (TTC) value of 1.5 µg/kg BW/day (Kroes et al., 2005) to assess dietary risk. Using the maximum chronic intake value (converted to µg/kg BW/day) from the 'Hispanic', 'children ages 1-6 years', 'children ages 7-12 years', 'children ages 1-2 years', 'children ages 3-5 years', and 'children ages 6-12 years' subgroups, the exposure to DvSSJ1 dsRNA from consumption of DP23211 grain was determined to be 750-fold below the TTC value. Additionally, there are known physical, chemical, enzymatic and molecular barriers to dietary dsRNA exposure and activity following ingestion and a history of safe consumption of dsRNA (see Section VI-A.3.a.

History of Safe Consumption of RNA). It is demonstrated later in this Petition that DvSSJ1 dsRNA activity is also specific to the *Diabrotica* species within the Chrysomelidae family (Table 67(Boeckman, 2019, in preparation)(Boeckman, 2019, in preparation), and an *in silico* analysis did not indicate a potential for off-target effects in humans (Volume 2, Appendix B).

VII.A.3.c.3. DvSSJ1 dsRNA Human Exposure Assessment Conclusion

Estimated exposure to DvSSJ1 dsRNA from consumption of DP23211 maize grain products is low. The estimated chronic exposure to DvSSJ1 dsRNA is orders of magnitude below the conservative threshold of toxicological concern. Additionally, a history of safe consumption of dsRNA, known barriers to dietary dsRNA exposure and activity, and the lack of identified off-target effects in humans are also noted for DvSSJ1 dsRNA. Therefore, consumption of DP23211 maize grain is expected to be as safe as conventional non-GE maize, especially when accounting for blending and processing.

See Appendix 8. Materials and Methods for Human Dietary Exposure Evaluation for further details regarding methods used for human dietary exposure.

VII-A.3.d. Livestock Exposure to DvSSJ1 dsRNA in DP23211 Maize

Utilizing the mean concentration of DvSSJ1 dsRNA in grain or forage from DP23211 maize (presented in Section VI-A.2. DvSSJ1 dsRNA Trait Expression Assessment), daily dietary exposure (DDE) to DvSSJ1 dsRNA from consumption of DP23211 maize grain and/or forage/silage were calculated for various livestock species using estimates of animal body weight (BW), daily feed intake, and grain and forage/silage inclusion rates specific for North America (OECD, 2013; corn, field). The following conservative total replacement scenarios were utilized:

- 100% DP23211 maize grain replacement for poultry (broiler, layer, turkey), swine (breeding, finishing), cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize forage/silage replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize grain and forage/silage combination replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb)

The estimated DDE to DvSSJ1 dsRNA with 100% DP23211 maize grain replacement was 0.00025 mg/kg BW/day for Broilers. The estimated DDEs to DvSSJ1 dsRNA with 100% DP23211 maize forage/silage or grain + forage/silage replacement were, respectively, 0.00034 and 0.00042 mg/kg BW/day for Dairy Cattle.

In practice, the actual livestock dietary exposures to DvSSJ1 dsRNA are expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus maize sources containing DP23211 DvSSJ1 dsRNA will be mixed with other maize grain sources potentially not

containing DvSSJ1 dsRNA, and (2) the estimates were highly conservative in their maize grain and/or forage/silage incorporation rates, not accounting for typical blending with other feedstuffs for adequate nutrient levels and least-cost formulations.

The low DvSSJ1 dsRNA maximum dietary exposure values (0.00025 to 0.00042 mg/kg BW), along with 1) a history of safe consumption of dsRNA and known physical, chemical, enzymatic and molecular barriers to dietary dsRNA exposure and activity following ingestion (VI-A.2.b Mammalian Barriers to Exposure to DvSSJ1 dsRNA Consumed in Food and Feed)(Roper, 2019)(Roper, 2019), 2) the highly specific and limited activity of DvSSJ1 dsRNA to the *Diabrotica* species within the Chrysomelidae family (Section IX-B.2. DvSSJ1 dsRNA Spectrum of Activity and Species Specificity), and 3) an *in silico* analysis that did not indicate a potential for off-target effects in poultry, swine, cattle, or sheep (Mirsky, 2019), indicate that consumption of DvSSJ1 dsRNA from DP23211 maize grain and/or forage/silage is not expected to pose a risk to animals.

The estimated livestock exposures to DvSSJ1 dsRNA from consumption of DP23211 maize grain and/or forage/silage is low. A history of safe consumption of dsRNA, known barriers to dietary dsRNA exposure and activity, and lack of identified off-target effects in livestock species are noted for DvSSJ1 dsRNA. Therefore, consumption of DP23211 maize grain and/or forage/silage is not expected to pose a risk to livestock, especially when accounting for market share and blending.

See Appendix 9. Materials and Methods for Livestock Dietary Exposure for further details regarding methods used for livestock dietary exposure.

VII-A.4. Conclusions on the Human and Livestock Safety of DvSSJ1 dsRNA in DP23211 Maize

The overall weight of evidence supports the conclusion that consumption of DvSSJ1 dsRNA by humans and animals in foods and feeds containing DP23211 maize is not expected to result in negative health effects in humans and animals. Nucleic acids, including dsRNA, are normal components of human and animal diets, and have a history of safe consumption in food and feed. The molecular target of DvSSJ1 dsRNA is specific to certain species within the *Diabrotica* genus of the Chrysomelidae family of Coleoptera, and has not been described in vertebrates (specificity is discussed in Section IX-B.2. DvSSJ1 dsRNA Spectrum of Activity and Species Specificity). Conservative estimates demonstrate low potential exposures to DvSSJ1 dsRNA for humans in food and animals in feed that are unlikely to result in cellular concentrations that could impact gene regulation, if a molecular target existed. Further, the well-characterized physical, enzymatic, biochemical and molecular barriers to exposure of ingested small RNAs will further reduce potential exposure to DvSSJ1 dsRNA consumed in food and feed from DP23211 maize. Collectively, the information presented herein indicates that consumption of DvSSJ1 dsRNA in

food or feed containing DP23211 maize is not expected to present a hazard to human or animal health, therefore supporting the overall safety assessment of DP23211 maize.

VII-B. Characterization of the IPD072Aa Protein in DP23211 Maize

In this section, we present protein characterization results via SDS-PAGE, western blot, peptide mapping, N-terminal amino acid sequence, and glycoprotein analysis. The IPD072Aa protein derived from DP23211 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation.

The allergenic potential of the IPD072Aa protein was evaluated using bioinformatic comparison of the of the IPD072Aa protein with known or putative protein allergen sequences; *in vitro* gastric and intestinal digestion models; IPD072Aa protein glycosylation status; and heat lability of the IPD072Aa protein. An acute toxicity study in mice and a bioinformatic comparison of the IPD072Aa amino acid sequence to known and putative protein toxins is also presented.

The results showed that the IPD072Aa protein is digested in SGF and SIF digestion analyses. Heat-treated IPD072Aa protein is shown to be inactive against WCR when incorporated in an artificial diet. The bioinformatic comparisons of the IPD072Aa protein sequence to known and putative allergen and toxin sequences showed that the IPD072Aa protein is unlikely to be allergenic or toxic for humans or animals. The acute oral toxicity assessment determined the LD50 of IPD072Aa protein to be greater than 2000 mg/kg. These data support the conclusion that the IPD072Aa protein in DP23211 maize is as safe as conventional maize for the food and feed supply and is unlikely to cause an adverse effect on humans or animals.

VII-B.1. IPD072Aa Source Organism

The IPD072Aa protein is derived from *Pseudomonas chlororaphis* (Schellenberger et al., 2016). *P. chlororaphis* is a naturally occurring, ubiquitous bacterium found in the environment that lacks known allergenic or toxic properties and has a history of safe use in agriculture (Anderson et al., 2018). Certain *Pseudomonas* species, including *P. chlororaphis,* have been used in agriculture as seed treatments, foliar-applied biopesticides for fungal and disease control, and as a gene source for GM crops. Over the past 30 years, the US EPA has registered several *Pseudomonas*-based biopesticides and granted exemptions from the requirements of a tolerance (e.g. 40 CFR 180-1114, 180.1145, 1802.1212) further demonstrating the history of safe use with this source organism (Anderson et al., 2018). The established ERA framework that is currently used to assess GM crops derived from *Bt* is robust and suitable for assessing plants expressing non-*Bt* proteins (Anderson et al., 2018). The IPD072Aa protein has been shown to be specific, with activity limited to within the order Coleoptera (Boeckman *et al.*, 2019).

VII-B.2. IPD072Aa Protein Trait Expression Assessment

The concentration levels of the IPD072Aa protein was evaluated in DP23211 maize using a robust field study.

Tissue samples were collected during the 2018 growing season at six sites in commercial maize-growing regions of the United States and Canada. A randomized complete block design with four blocks was utilized at each site. The following tissue samples were collected: root (V6, V9, R1, R4, and R6 growth stages), leaf (V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), forage (R4 growth stage), whole plant (R1 and R6 growth stages), and grain (R6 growth stage). Concentrations of the IPD072Aa protein was determined using quantitative enzyme-linked immunosorbent assays (ELISA). The IPD072Aa ELISA was internally validated to demonstrate method suitability (Appendix 6. Methods for Determination of DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI Protein Concentrations).

The results of the IPD072Aa protein concentration analysis (means, ranges, and standard deviations) are summarized across sites in Table 19. Individual sample results below the LLOQ were assigned a value equal to half of the LLOQ for calculation purposes.

Additional details regarding analytical methods and calculations for trait expression analysis are provided in Appendix 6. Methods for Determination of DvSSJ1 dsRNA and IPD072Aa, PAT, and

PMI Protein Concentrations.

Table 19. Across Sites Summary of IPD072Aa Protein Concentrations in DP23211 Maize

Tissue (Growth Stage)	ng IPD072Aa/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	Range	Standard Deviation	Sample LLOQ	
DP23211 Maize					
Root (V6)	25	4.2 - 60	16	0.11	0/24
Root (V9)	19	3.6 - 84	23	0.11	0/24
Root (R1)	21	7.5 - 51	8.8	0.11	0/24
Root (R4)	24	6.6 - 42	8.5	0.11	0/24
Root (R6)	31	0.93 - 72	21	0.11	0/24
Leaf (V9)	13	2.8 - 39	11	0.054	0/24
Leaf (R1)	16	5.5 - 33	7.6	0.054	0/24
Leaf (R4)	10	5.8 - 15	2.5	0.054	0/24
Leaf (R6)	1.6[a]	<0.054 - 10	2.5[a]	0.054	1/24
Pollen (R1)	0.65	0.14 - 1.3	0.38	0.11	0/24
Whole Plant (R1)	7.9	2.4 - 14	2.3	0.018	0/24
Whole Plant (R6)	11	1.7 - 24	7.5	0.018	0/24
Forage (R4)	16	6.0 - 28	7.2	0.018	0/24
Grain (R6)	2.1	0.51 - 4.8	1.2	0.027	0/24

Note: Growth stages (Abendroth et al., 2011). Lower limit of quantification (LLOQ) in ng/mg tissue dry weight.
[a] Some, but not all, sample results were below the LLOQ. A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.

VII-B.3. Safety Assessment of IPD072Aa Protein in DP23211 Maize

VII-B.3.a. Amino Acid Sequence of the IPD072Aa Protein in DP23211 Maize

The deduced amino acid sequence from the translation of the *ipd072Aa* gene is 86 amino acids in length and has a molecular weight of approximately 10 kDa (Figure 22)

```
  1   MGITVTNNSS NPIEVAINHW GSDGDTSFFS VGNGKQETWD RSDSRGFVLS
 51   LKKNGAQHPY YVQASSKIEV DNNAVKDQGR LIEPLS*
```

Figure 22. Deduced Amino Acid Sequence of the IPD072Aa Protein

The deduced amino acid sequence from the translation of the *ipd072Aa* gene from plasmid PHP74643. The asterisk (*) indicates the translational stop codon. The full-length protein is 86 amino acids in length and has a molecular weight of approximately 10 kDa.

VII-B.3.b. Function and Activity of the IPD072Aa Protein in DP23211 Maize

The IPD072Aa protein, encoded by the *ipd072Aa* gene, confers control of certain coleopteran pests when expressed in plants by causing disruption of the midgut epithelium. The *ipd072Aa* gene was identified and cloned from a *Pseudomonas chlororaphis* strain that was cultured from a soil sample (Schellenberger et al., 2016).

Additional information regarding mode of action and specificity is provided in Sections IX-C.1. IPD072Aa Mode of Action. and IX-C.2. IPD072Aa Spectrum of Activity and Species Specificity.

VII-B.3.c. Equivalence of the IPD072Aa Protein Derived from DP23211 Maize and Microbial Systems

The IPD072Aa protein was partially purified from DP23211 maize whole plant tissue using ammonium sulfate precipitation and immuno-affinity chromatography.

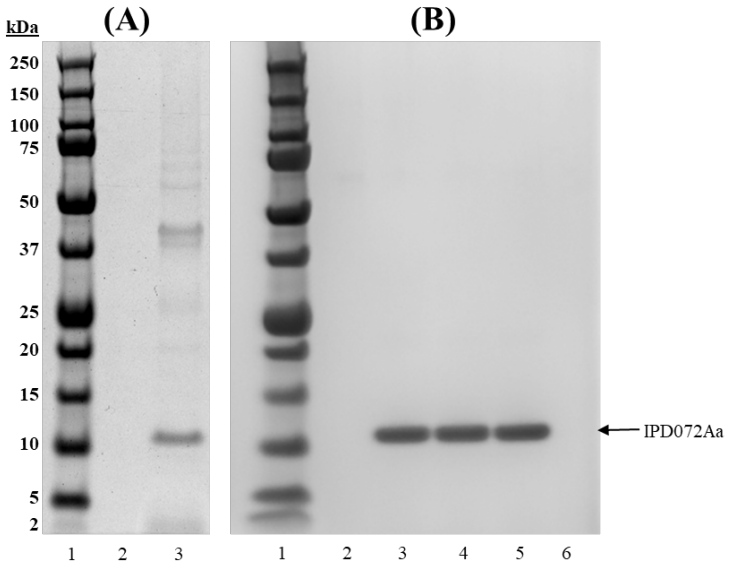
In order to have sufficient amounts of purified IPD072Aa protein for the multiple studies required to assess its safety, IPD072Aa protein was expressed in an *Escherichia coli* protein expression system as a fusion protein with an N-terminal histidine tag. The microbially derived protein was purified using nickel affinity chromatography, and the histidine tag was cleaved with immobilized trypsin and then removed using nickel affinity chromatography (Carlson et al., 2019).

The equivalence in biochemical characteristics between the microbially derived IPD072Aa protein and the DP23211 maize-expressed IPD072Aa protein was characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, western blot analysis, peptide mapping by mass spectrometry, N-terminal amino acid sequencing, and glycoprotein analysis. The results demonstrated that the IPD072Aa protein derived from DP23211 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation. The microbially derived IPD072Aa protein was demonstrated to be equivalent to the DP23211 maize-derived IPD072Aa protein for use in safety testing.

VII-B.3.c.1. SDS-PAGE Analysis

Samples of IPD072Aa protein purified from DP23211 maize whole plant tissue and microbially derived IPD072Aa protein purified from a microbial expression system were analyzed separately by SDS-PAGE. As expected, all IPD072Aa protein samples migrated as a predominant band consistent with the expected molecular weight of approximately 10 kDa (Carlson *et al.*, 2019), as shown in Figure 23.

Additional details regarding SDS-PAGE analytical methods are provided in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.



Panel	Lane	Sample Identification
A	1	Pre-stained Protein Molecular Weight Marker
	2	1X LDS Sample Buffer Blank
	3	DP23211 Maize-Derived IPD072Aa Protein
B	1	Pre-stained Protein Molecular Weight Marker
	2	1X LDS Sample Buffer Blank
	3	Microbially Derived IPD072Aa Protein (1 µg)
	4	Microbially Derived IPD072Aa Protein (1 µg)
	5	Microbially Derived IPD072Aa Protein (1 µg)
	6	1X LDS Sample Buffer Blank

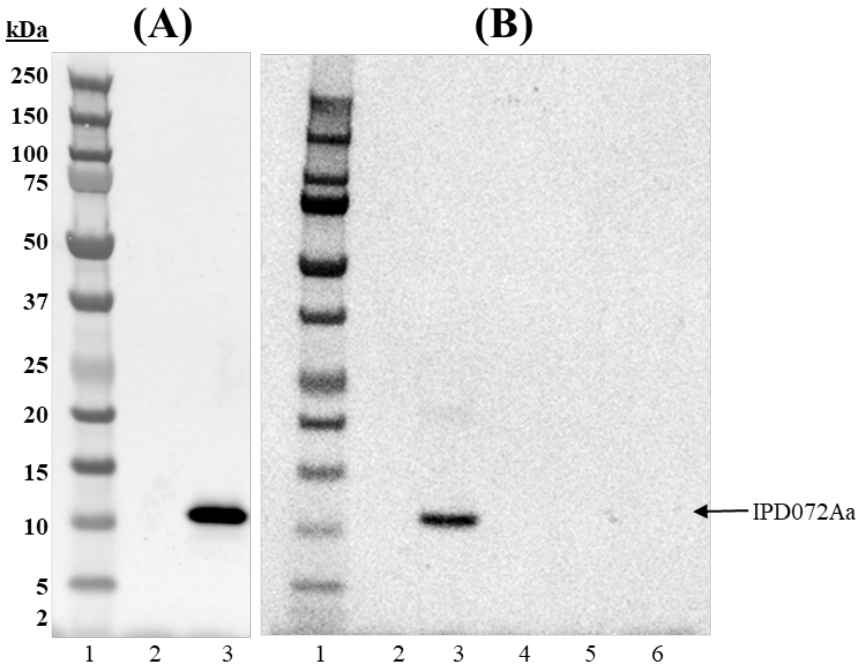
Note: kilodalton (kDa), microgram (µg). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 23. SDS-PAGE Analysis of IPD072Aa Protein

VII-B.3.c.2.Western Blot Analysis

Samples of IPD072Aa protein purified from DP23211 maize whole plant tissue and IPD072Aa protein purified from a microbial expression system were analyzed separately by Western blot. As expected, all IPD072Aa protein samples were immunoreactive to an IPD072Aa polyclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 10 kDa (Carlson *et al.*, 2019), as shown in Figure 24.

Additional details regarding Western blot analytical methods are provided in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.



Panel	Lane	Sample Identification
A	1	Pre-stained Protein Molecular Weight Marker
	2	1X LDS Sample Buffer Blank
	3	DP23211 Maize-Derived IPD072Aa Protein
B	1	Pre-stained Protein Molecular Weight Marker
	2	1X LDS Sample Buffer Blank
	3	Microbially Derived IPD072Aa Protein (5 ng)
	4	1X LDS Sample Buffer Blank
	5	1X LDS Sample Buffer Blank
	6	1X LDS Sample Buffer Blank

Note: kilodalton (kDa), nanogram (ng). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 24. Western Blot Analysis of IPD072Aa Protein

VII-B.3.c.3 .Mass Spectrometry Peptide Mapping Analysis

Samples of IPD072Aa protein purified from DP23211 maize whole plant tissue and IPD072Aa protein purified from a microbial expression system were analyzed separately by SDS-PAGE. Protein bands were stained with Coomassie stain reagent, and the band containing IPD072Aa protein was excised for each sample.

The excised IPD072Aa protein bands derived from DP23211 maize were digested with trypsin and chymotrypsin. Digested samples were analyzed using liquid chromatography-mass spectrometry (LC-MS). The mass data was used to search and match the peptides from the expected IPD072Aa protein sequence. The identified matched peptides account for 65% (56/86) of the expected IPD072Aa amino acid sequence (Table 20, Table 21, Table 22and Figure 25).

The microbially derived IPD072Aa protein bands were digested with chymotrypsin. Digested samples were analyzed using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS). For some digested peptides, MALDI tandem mass spectrometry (MALDI MS/MS) was performed for peptide fragmentation analysis (i.e., partial sequencing). The MS and MS/MS spectra were combined, and the data was used to search and match the peptides from the expected IPD072Aa protein sequence. The identified matched peptides account for 100% of the expected IPD072Aa amino acid sequence (Carlson et al., 2019), as shown in Table 23 and Figure 26.

Additional details regarding peptide mapping analytical methods are provided in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.

Table 20. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DP23211 Maize-Derived IPD072Aa Protein Using LC-MS Analysis

Protease	% Coverage	Combined % Coverage
Trypsin	53	65
Chymotrypsin	20	

Table 21. Tryptic Peptides of DP23211 Maize-Derived IPD072Aa Protein Identified Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
36-41	833.3607	833.3668	QETWDR
46-52	762.4591	762.4640	GFVLSLK
54-67	1548.7241	1548.7321	NGAQHPYYVQASSK
68-76	1000.5124	1000.5189	IEVDNNAVK
68-80	1456.7226	1456.7270	IEVDNNAVKDQGR
81-86	670.3866	670.3901	LIEPLS

[a] The experimental mass is the uncharged mass calculated from the mass to charge ratio of the observed ion.

[b] The theoretical mass is the *in silico* generated mass that matches closest to the experimental mass.

Table 22. Chymotryptic Peptides of DP23211 Maize-Derived IPD072Aa Protein Identified Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
30-39	1104.5143	1104.5200	SVGNGKQETW
40-47	938.4152	938.4206	DRSDSRGF

[a] The experimental mass is the uncharged mass calculated from the mass to charge ratio of the observed ion.
[b] The theoretical mass is the *in silico* generated mass that matches closest to the experimental mass.

1 MGITVTNNSS NPIEVAINHW GSDGDTSFF**S VGNGKQETWD RSDSRGFVLS**
51 **LK**K**NGAQHPY YVQASSKIEV DNNAVKDQGR LIEPLS**

Gray shading	Gray-shaded type indicates DP23211 maize-derived IPD072Aa peptides identified using LC-MS analysis.
Amino acid residue abbreviations	alanine (A), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Figure 25. Amino Acid Sequence of DP23211 Maize-Derived IPD072Aa Protein Indicating Tryptic and Chymotryptic Peptides Identified Using LC-MS Analysis

Table 23. Chymotryptic Peptides of Microbially Derived IPD072Aa Protein Identified Using MALDI-MS Analysis

IPD072Aa Amino Acid Residue Position	IPD072Aa Theoretical Peptide Mass (M+H)	IPD072Aa Observed Peptide Mass (M+H) (input)	Identified Peptide Sequence
1 - 30	3247.47	3247.33	HMGITVTNNSSNPIEVAINHWGSDGDTSFF
31 - 40	1105.52	1105.51	SVGNGKQETW
31 - 48	2025.93	2025.91	SVGNGKQETWDRSDSRGF
31 - 50	2238.08	2238.05	SVGNGKQETWDRSDSRGFVL
41 - 48	939.42	939.42	DRSDSRGF
41 - 50	1151.57	1151.56	DRSDSRGFVL
49 - 61	1454.80	1454.79	VLSLKKNGAQHPY
51 - 61	1242.65	1242.64	SLKKNGAQHPY
62 - 87	2873.49	2873.46	YVQASSKIEVDNNAVKDQGRLIEPLS

Note: alanine (A), arginine (R), asparagine (N), aspartic acid (D), glutamic acid (E), glutamine (Q), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

HMGITVTNNSSNPIEVAINHWGSDGDTSFFSVGNGKQETWDRSDSRGFVLSLKKNGAQHPYYVQ ASSKIEVDNNAVKDQGRLIEPLS

Gray shading	Gray-shaded type indicates microbially derived IPD072Aa peptides identified using MALDI-MS analysis.
Amino acid residue abbreviations	alanine (A), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Figure 26. Amino Acid Sequence of Microbially Derived IPD072Aa Protein Indicating Chymotryptic Peptides Identified Using MALDI-MS Analysis

VII-B.3.c.4. N-Terminal Amino Acid Sequence Analysis

Two samples of IPD072Aa protein purified from DP23211 maize whole plant tissue were analyzed by SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membrane. Protein bands were stained using GelCode Blue stain reagent, and the band containing IPD072Aa protein was excised for each sample. Both bands were analyzed as a single sample using Edman sequencing to determine the N-terminal amino acid sequence. The analysis obtained a primary sequence (GITVTNNSSN) matching amino acid residues 2-11 of the deduced IPD072Aa protein sequence (Table 24), indicating the N-terminal methionine was absent as expected (Dummitt et al., 2003; Sherman et al., 1985).

Samples of IPD072Aa protein purified from a microbial expression system were directly analyzed using Edman sequencing to determine the N-terminal amino acid sequence. The analysis obtained the same primary sequence (HMGITVTNNS), matching amino acid residues 1-10 of the expected sequence of the microbially derived IPD072Aa protein (Carlson *et al.*, 2019).

Additional details regarding N-terminal amino acid sequencing analytical methods are provided in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.

Table 24. N-Terminal Amino Acid Sequence Analysis of IPD072Aa Protein

Description		Amino Acid Sequence
DP23211 Maize-Derived IPD072Aa Protein	Theoretical Sequence	M – G – I – T – V – T – N – N – S – S – N
	Observed Sequence	G – I – T – V – T – N – N – S – S – N
Microbially Derived IPD072Aa Protein	Theoretical Sequence	H – M – G – I – T – V – T – N – N – S
	Observed Sequence (Tox Lot PCF-0037-AP)	H – M – G – I – T – V – T – N – N – S
	Observed Sequence (Tox Lot PCF-0040)	H – M – G – I – T – V – T – N – N – S

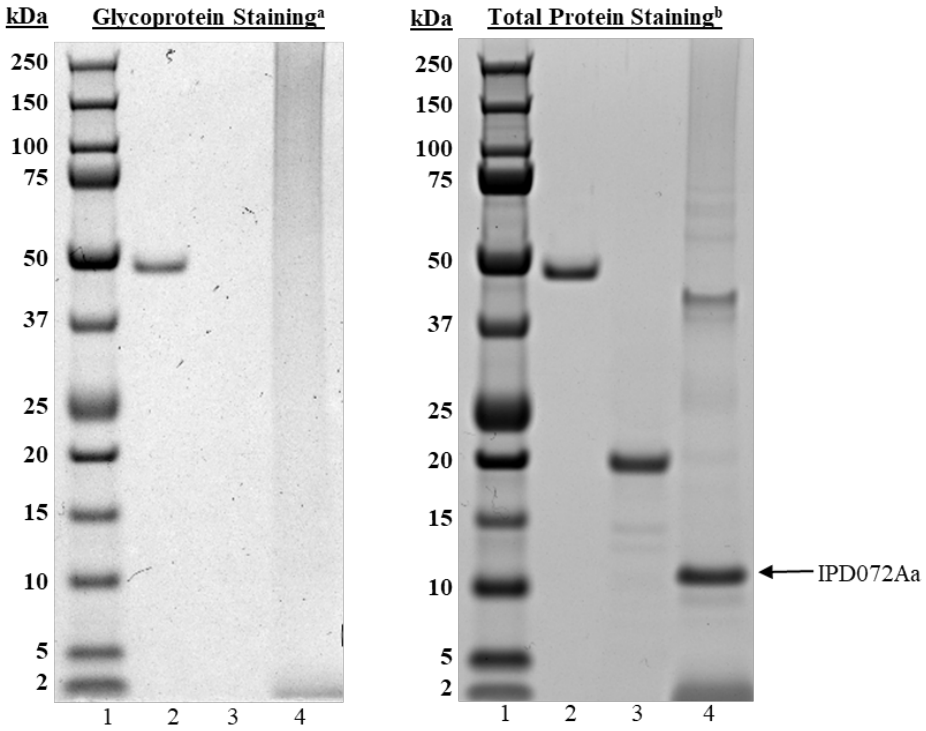
Note: The N-terminal methionine in the detected primary sequence for DP23211 maize-derived IPD072Aa protein was absent as expected. Asparagine (N), glycine (G), histidine (H), isoleucine (I), methionine (M), serine (S), threonine (T), and valine (V).

VII-B.3.c.5. Glycoprotein Analysis

Samples of IPD072Aa protein purified from DP23211 maize whole plant tissue and IPD072Aa protein purified from a microbial expression system were analyzed separately by SDS-PAGE. Each gel also included a positive control (horseradish peroxidase) and negative control (soybean trypsin inhibitor). The gels were then stained using a Pierce Glycoprotein Staining Kit to visualize any glycoproteins. The gels were imaged and then stained with GelCode Blue stain reagent to visualize all protein bands.

Glycosylation was not detected for any of the IPD072Aa protein samples (Figure 27 and Figure 28). The horseradish peroxidase positive control was clearly visible as a stained band. The soybean trypsin inhibitor negative control was not stained by the glycoprotein stain.

Additional details regarding glycoprotein analytical methods are provided in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[c]
2	Positive Control: Horseradish Peroxidase (1.0 µg)
3	Negative Control: Soybean Trypsin Inhibitor (1.0 µg)
4	DP23211 Maize-Derived IPD072Aa Protein

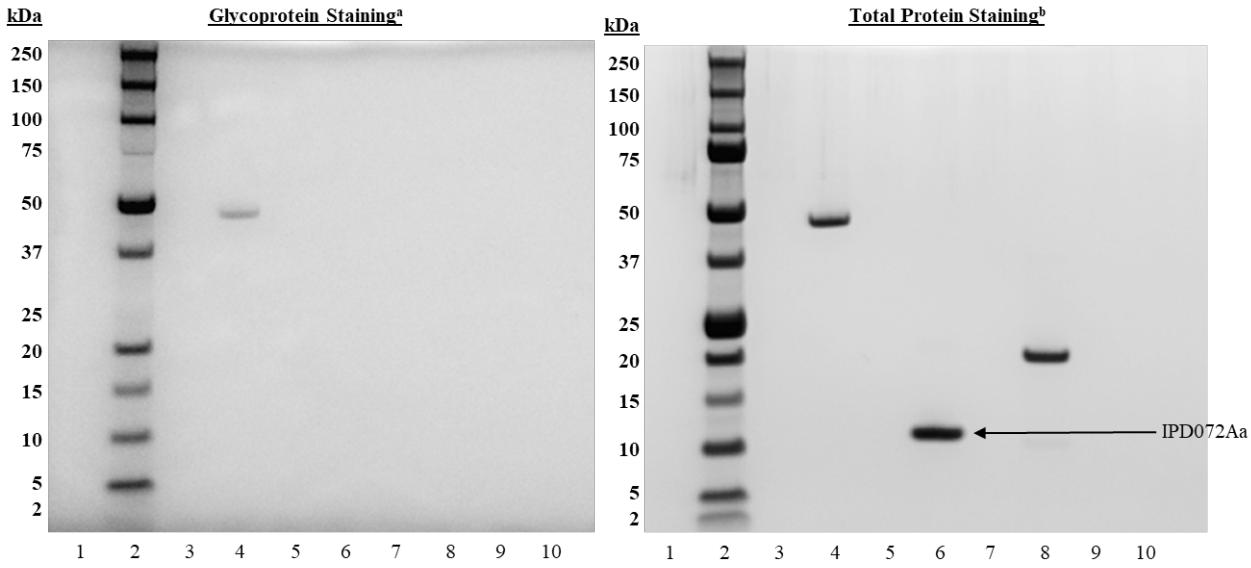
Note: kilodalton (kDa), microgram (µg).

[a] Gel was stained with glycoprotein staining reagent.

[b] Gel was stained with glycoprotein staining reagent followed by staining with Coomassie Blue Reagent for total proteins.

[c] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 27. Glycosylation Analysis of DP23211 Maize-Derived IPD072Aa Protein



Lane	Sample Identification
1	1X LDS Sample Buffer Blank
2	Pre-stained Protein Molecular Weight Marker[c]
3	1X LDS Sample Buffer Blank
4	Positive Control: Horseradish Peroxidase (1 µg)
5	1X LDS Sample Buffer Blank
6	Microially Derived IPD072Aa Protein (1 µg)
7	1X LDS Sample Buffer Blank
8	Negative Control: Soybean Trypsin Inhibitor (1 µg)
9	1X LDS Sample Buffer Blank
10	1X LDS Sample Buffer Blank

Note: kilodalton (kDa), microgram (µg).

[a] Gel was stained with glycoprotein staining reagent.

[b] Gel was stained with glycoprotein staining reagent followed by staining with Coomassie Blue Reagent for total proteins.

[c] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 28. Glycosylation Analysis of Microbially Derived IPD072Aa Protein

VII-B.3.d. Allergenicity and Toxicity Analyses of the IPD072Aa Protein in DP23211 Maize

A weight-of-evidence approach was applied to determine the allergenic and toxic potential of the IPD072Aa protein expressed in DP23211 maize, including an assessment of the following: the history of safe use of the source organism, a bioinformatic comparison of the amino acid sequence of IPD072Aa protein to known or putative allergenic and toxic proteins, evaluation of the stability of the IPD072Aa protein using *in vitro* gastric and intestinal digestion models, determination of the glycosylation status of the IPD072Aa protein, an evaluation of the heat lability of IPD072Aa protein via a sensitive insect bioassay, and an evaluation of acute toxicity in mice following oral exposure to IPD072Aa protein. A summary of the safety assessment for IPD072Aa protein was published by Carlson *et al.* (2019).

VII-B.3.d.1. IPD072Aa Protein Source and History of Safe Use

The *ipd072Aa* gene that encodes the IPD072Aza protein was identified and cloned from a *Pseudomonas chlororaphis* strain that was isolated from a soil sample (Schellenberger et al., 2016). *Pseudomonas chlororaphis* is a rod-shaped, aerobic, Gram-negative bacterium that is ubiquitous in soil, has a history of safe use in agriculture and in food and feed crops, and is not known to be allergenic, toxic, or pathogenic to humans, animals, or livestock (Anderson et al., 2018).

VII-B.3.d.2. Bioinformatic Analysis of IPD072Aa Protein Homology to Known or Putative Allergens

Assessing newly expressed proteins for potential cross-reactivity with known or putative allergens is an important part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically-modified plant products (Codex Alimentarius Commission, 2003). In this study, a bioinformatic assessment of the IPD072Aa protein sequence for potential cross-reactivity with known or putative allergens was conducted according to relevant guidelines (Codex Alimentarius Commission, 2003; FAO/WHO, 2001a).

Two separate searches for the IPD072Aa protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2019 database (January 2019) available at http://comparedatabase.org. This peer-reviewed database is a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins, and Bioinformatics (PATB) Committee and is comprised of 2,081 sequences. The first search used the IPD072Aa protein sequence as the query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the allergen sequences. The search was conducted using default parameters, except the *E*-score threshold was set to 10^{-4}. An *E*-score threshold of 10^{-4} has been shown to be an appropriate value for allergenicity searches (Mirsky et al., 2013). The generated alignments were examined to identify any that are a length of 80 or greater and possess a sequence identity of ≥ 35%. The second

search used an in-house Perl script (runLinearEpitopeScreen.pl) to identify any contiguous 8-residue identical matches between the IPD072Aa protein sequence and the allergen sequences.

Results of the search of the IPD072Aa protein sequence against the COMPARE database of known and putative allergen sequences found no alignments that were a length of 80 or greater with a sequence identity of ≥ 35%. No contiguous 8-residue matches between the IPD072Aa protein sequence and the allergen sequences were identified in the second search. Taken together, the comparisons of the IPD072Aa protein sequence to the allergen sequences showed that there is no apparent allergenicity concern regarding the IPD072Aa protein

Methods and bioinformatic output are presented in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.

VII B.3.d.3. Thermolability Analysis of IPD072Aa Protein

Thermal stability of the IPD072Aa protein was characterized by determining the biological activity of heat-treated IPD072Aa protein incorporated in an artificial diet fed to WCR. Purified IPD072Aa protein was incubated at various temperatures for approximately 30 minutes before incorporation into the artificial diet. WCR larvae were exposed via oral ingestion to the diets in a 7-day bioassay. A positive control diet containing unheated IPD072Aa protein and a bioassay control diet containing water were included in the bioassay to verify assay performance. After seven days, statistical analyses were conducted to evaluate WCR mortality of the heat-treated test groups relative to the unheated test group.

The results demonstrated that IPD072Aa protein autoclaved for approximately 30 minutes at a targeted temperature of 121 °C and 20 psi was inactive against WCR when incorporated in an artificial insect diet (Table 25).

Additional details regarding thermolability analytical methods are provided in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.

Table 25. Biological Activity of Heat-Treated IPD072Aa Protein in Artificial Diet Fed to Western Corn Rootworm

Treatment	Treatment Description	Test Dosing Solution Incubation Condition	Total Number of Observations[a]	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	NA	29	8	27.6	--
2	Test Diet	Unheated	22	20	90.9	--
3	Test Diet	25 °C	29	26	89.7	1.0000
4	Test Diet	50 °C	26	23	88.5	1.0000
5	Test Diet	60 °C	28	24	85.7	0.6825
6	Test Diet	95 °C	24	20	83.3	0.6672
7	Test Diet	121 °C (autoclaved)	29	4	13.8	<0.0001[b]

Note: Test diets contained a targeted concentration of 50 ng IPD072Aa protein per mg diet wet weight. Not applicable (NA); the bioassay control diet was not incubated.

[a] Organisms counted as missing during the bioassay, or wells containing more than one organism, were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed in comparison to Treatment 2.

VII-B.3.d.4. Digestibility Analysis of IPD072Aa protein with Simulated Gastric Fluid

Simulated gastric fluid (SGF) containing pepsin at pH ~1.2 was used to assess the susceptibility of the IPD072Aa protein to proteolytic digestion by pepsin *in vitro*. IPD072Aa protein was incubated in SGF for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 minutes. A positive control (bovine serum albumin) and a negative control (β-lactoglobulin) were included in the assay and were incubated in SGF for 0, 1, and 60 minutes. After incubation in SGF, the samples were analyzed by SDS-PAGE. Coomassie-based stain or western blot was used to detect protein bands.

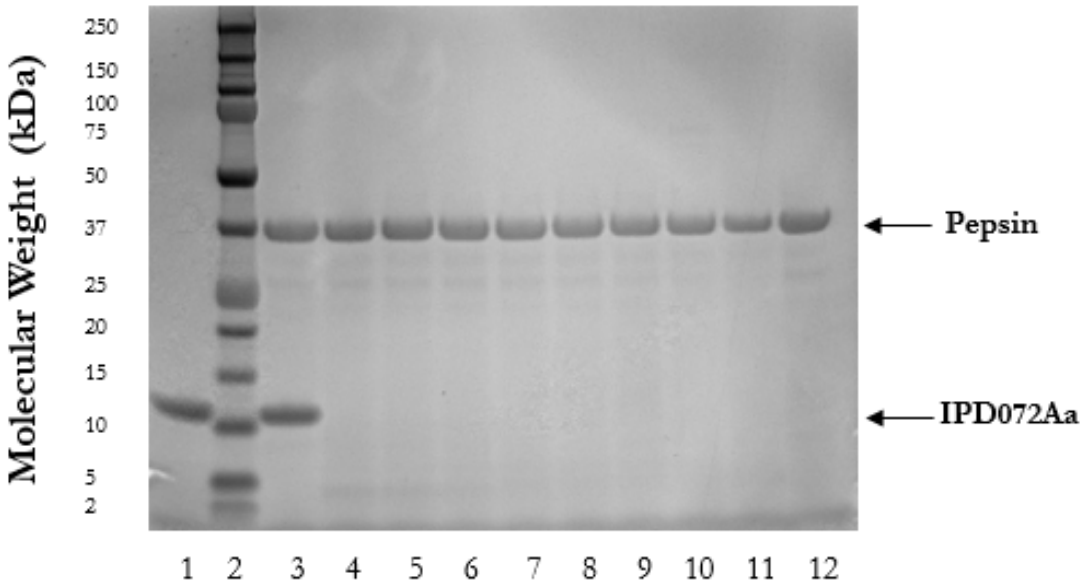
A summary of the SGF results is provided in Table 26. The IPD072Aa protein was rapidly digested (within 0.5 minutes) in SGF as demonstrated by both SDS-PAGE and western blot analysis (Figure 29 and Figure 30, respectively). The bovine serum albumin control substance disappeared rapidly (less than one minute) in SGF and the β-lactoglobulin control persisted through the 60-minute timecourse, verifying that the assay performed as expected.

Additional details regarding SGF analytical methods are provided in Appendix 5.

Table 26. Summary of IPD072Aa Protein *In Vitro* Pepsin Resistance Assay Results

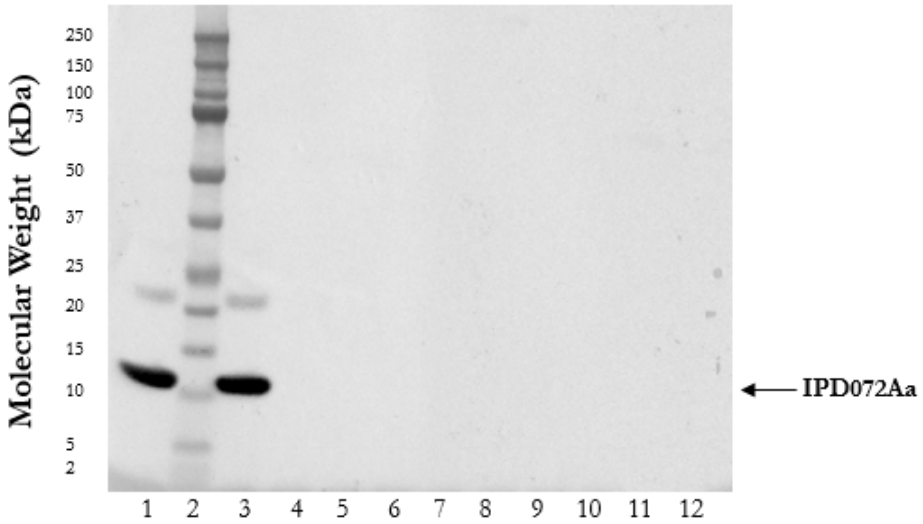
Protein	Approximate Molecular Weight (kDa)	Digestion Time Determined by SDS-PAGE (minutes)	Digestion Time Determined by Western Blot (minutes)
IPD072Aa Protein	10	≤ 0.5	≤ 0.5
Bovine Serum Albumin (positive control)	66	≤ 1	NA
β-Lactoglobulin (negative control)	18	> 60	NA

Note: Kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and not applicable (NA).



Lane	Sample Descriptions
1	IPD072Aa protein in water, time 0
2	Pre-stained protein molecular weight marker
3	IPD072Aa protein in SGF, time 0
4	IPD072Aa protein in SGF, 0.5 minutes
5	IPD072Aa protein in SGF, 1 minute
6	IPD072Aa protein in SGF, 2 minutes
7	IPD072Aa protein in SGF, 5 minutes
8	IPD072Aa protein in SGF, 10 minutes
9	IPD072Aa protein in SGF, 20 minutes
10	IPD072Aa protein in SGF, 30 minutes
11	IPD072Aa protein in SGF, 60 minutes
12	SGF control, 60 minutes

Figure 29. SDS-PAGE Analysis of IPD072Aa Protein in Simulated Gastric Fluid Digestion Time-Course



Lane	Sample Descriptions
1	IPD072Aa protein in water, time 0
2	Pre-stained protein molecular weight marker
3	IPD072Aa protein in SGF, time 0
4	IPD072Aa protein in SGF, 0.5 minutes
5	IPD072Aa protein in SGF, 1 minute
6	IPD072Aa protein in SGF, 2 minutes
7	IPD072Aa protein in SGF, 5 minutes
8	IPD072Aa protein in SGF, 10 minutes
9	IPD072Aa protein in SGF, 20 minutes
10	IPD072Aa protein in SGF, 30 minutes
11	IPD072Aa protein in SGF, 60 minutes
12	SGF control, 60 minutes

Note: Kilodalton (kDa), simulated gastric fluid (SGF), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 30. Western Blot Analysis of IPD072Aa Protein in Simulated Gastric Fluid Digestion Time-Course

VII-B.3.d.5. Digestibility Analysis of IPD072Aa Protein with Simulated Intestinal Fluid

Simulated intestinal fluid (SIF) containing pancreatin at ~pH 7.5 was used to assess the susceptibility of the IPD072Aa protein to proteolytic digestion by pancreatin *in vitro*. IPD072Aa protein was incubated in SIF for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 minutes. Two control proteins (bovine serum albumin and β-lactoglobulin) were included in the assay and were incubated in SIF for 0, 1, and 60 minutes. After incubation in SIF, the samples were analyzed by SDS-PAGE. Coomassie-based stain or western blot was used to detect protein bands.

A summary of the SIF assay results is provided in Table 27. The IPD072Aa protein was digested in SIF within 20 minutes as demonstrated by both SDS-PAGE and western blot analysis (Figure 31 and Figure 32, respectively). The β-lactoglobulin control substance disappeared rapidly (less than

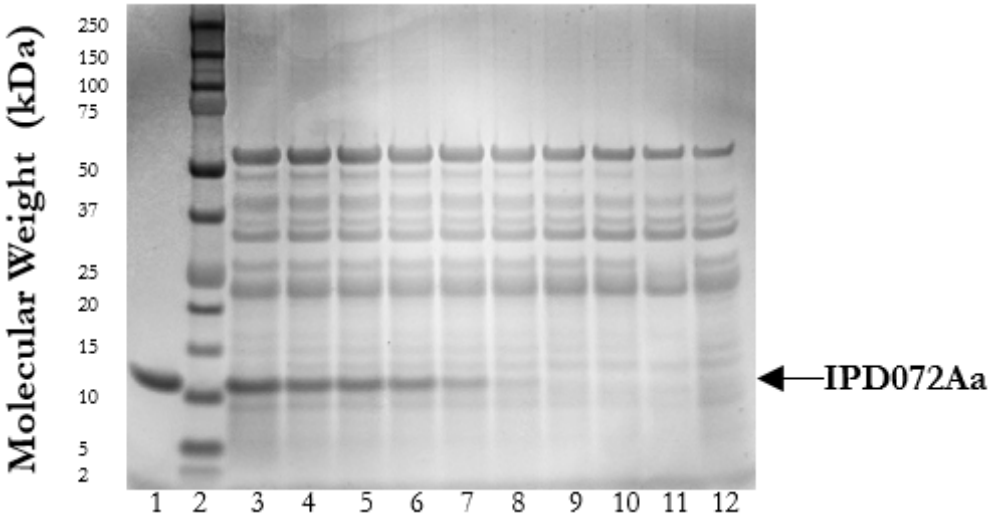
one minute) in SIF and the bovine serum albumin control persisted through the 60-minute timecourse, verifying that the assay performed as expected.

Additional details regarding SIF analytical methods are provided in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.

Table 27. Summary of IPD072Aa Protein *In Vitro* Pancreatin Resistance Assay Results

Protein	Approximate Molecular Weight (kDa)	Digestion Time Determined by SDS-PAGE (minutes)	Digestion Time Determined by Western Blot (minutes)
IPD072Aa Protein	10	≤ 20	≤ 20
Bovine Serum Albumin (control)	66	> 60	NA
β-Lactoglobulin (control)	18	≤ 1	NA

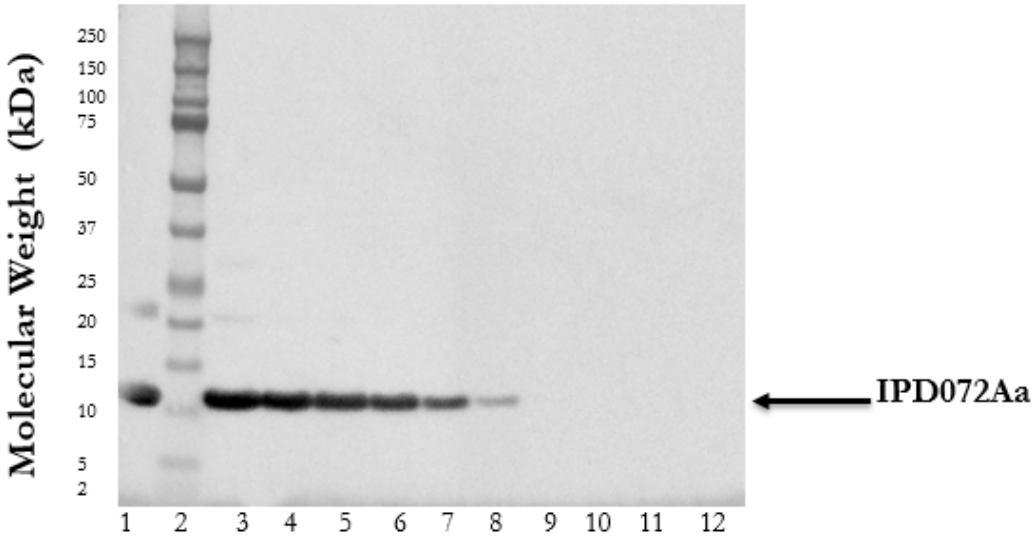
Note: Kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), not applicable (NA).



Lane	Sample Descriptions
1	IPD072Aa protein in water, time 0
2	Pre-stained protein molecular weight marker
3	IPD072Aa protein in SIF, time 0
4	IPD072Aa protein in SIF, 0.5 minutes
5	IPD072Aa protein in SIF, 1 minute
6	IPD072Aa protein in SIF, 2 minutes
7	IPD072Aa protein in SIF, 5 minutes
8	IPD072Aa protein in SIF, 10 minutes
9	IPD072Aa protein in SIF, 20 minutes
10	IPD072Aa protein in SIF, 30 minutes
11	IPD072Aa protein in SIF, 60 minutes
12	SIF control, 60 minutes

Note: Kilodalton (kDa), simulated intestinal fluid (SIF), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 31. SDS-PAGE Analysis of IPD072Aa Protein in Simulated Intestinal Fluid Digestion Time-Course



Lane	Sample Descriptions
1	IPD072Aa protein in water, time 0
2	Pre-stained protein molecular weight marker
3	IPD072Aa protein in SIF, time 0
4	IPD072Aa protein in SIF, 0.5 minutes
5	IPD072Aa protein in SIF, 1 minute
6	IPD072Aa protein in SIF, 2 minutes
7	IPD072Aa protein in SIF, 5 minutes
8	IPD072Aa protein in SIF, 10 minutes
9	IPD072Aa protein in SIF, 20 minutes
10	IPD072Aa protein in SIF, 30 minutes
11	IPD072Aa protein in SIF, 60 minutes
12	SIF control, 60 minutes

Note: Kilodalton (kDa), simulated intestinal fluid (SIF), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 32. Western Blot Analysis of IPD072Aa Protein in Simulated Intestinal Fluid Digestion Time-Course

VII-B.3.d. 6.IPD072Aa Protein Glycoprotein Analysis

As stated previously in Section VII-B.3.c.5. Glycoprotein Analysis, the results from glycoprotein staining analysis confirmed the absence of glycosylation for IPD072Aa protein isolated and purified from DP23211 maize tissue.

VII-B.3.d.7. Bioinformatic Analysis of IPD072Aa Protein Homology to Known or Putative Toxins

Assessing newly expressed proteins for potential toxicity is a critical part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically modified plant products (Codex Alimentarius Commission, 2003). The potential toxicity of the IPD072Aa protein was assessed by comparison of its sequence to the sequences in an internal Corteva Agriscience (internal) toxin database. The internal toxin database is a subset of sequences found in

UniProtKB/Swiss-Prot (https://www.uniprot.org/). To produce the internal toxin database, the proteins in UniProtKB/Swiss-Prot are filtered for molecular function by keywords that could imply toxicity or adverse health effects (*e.g.*, toxin, hemagglutinin, vasoactive, etc.). The DuPont Pioneer toxin database is updated annually. The search between the IPD072Aa protein sequence and protein sequences in the internal toxin database was conducted with BLASTP using default parameters, except that low complexity filtering was turned off, the *E*-value threshold was set to 10^{-4}, and unlimited alignments were returned.

No alignments with an E-value ≤ 10^{-4} were returned between the IPD072Aa protein sequence and any protein sequence in the internal toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the IPD072Aa protein.

Methods and bioinformatic output are presented in Appendix 5. (Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins).

VII-B.3.d.8. Evaluation of the Acute Toxicity of IPD072Aa Protein

A study was conducted to evaluate the acute toxicity of the test substance, IPD072Aa protein, in groups of 6 male and 6 female Crl:CD1(ICR) mice following oral exposure at a dose of 2000 mg/kg. IPD072Aa protein and Bovine Serum Albumin (BSA) protein were each reconstituted in deionized water. A vehicle control, BSA control and IPD072Aa test substance formulations were administered orally by gavage. The mice were fasted prior to and throughout the dosing procedure.

Body weights were evaluated on test days 1 (prefast and shortly prior to dose administration), 2, 3, 5, 8, and 15. Clinical signs were evaluated before and after dosing on test day 1 and daily thereafter. On test day 15, all mice were euthanized and given a gross pathological examination.

All animals survived to scheduled euthanasia. There were no clinical abnormalities or overall (test day 1-15) losses in body weight among any of the animals tested. No gross lesions were present at necropsy.

Under the conditions of this study, intragastric exposure of IPD072Aa protein to male and female mice at 2000 mg/kg did not result in mortality or other evidence of acute oral toxicity, based on evaluation of body weight, clinical signs, and gross pathology. Therefore, the LD_{50} of IPD072Aa protein was determined to be greater than 2000 mg/kg.

Methods for the evaluation of acute toxicity of the IPD072Aa Protein are presented in Volume 2, Appendix G, Section G8.

VII-B.3.e. Human Exposure Assessment of IPD072Aa Protein in DP23211 Maize

The intended use(s) and degree of exposure can be considered in assessing the safety of a GE crop. This consideration includes the effect(s) (if any) of the level of the food or food product in the diet, patterns of dietary consumption, and the defining characteristics of populations that consume the crop of interest (OECD, 1993).

The levels of expression of the IPD072Aa protein was measured in edible tissues and is reported in Table 19. These values were then used to estimate potential exposure to humans and livestock as reported below.

VII-B.3.e.1. IPD072Aa Protein Human Exposure Assessment Methods

Dietary exposure to the IPD072Aa protein was calculated utilizing the mean concentrations of the IPD072Aa protein in DP23211 maize grain presented in Table 19, along with consumption data from the Dietary Exposure Evaluation Model – Food Commodity Intake Database (DEEM™ - FCID), Version 4.02 (DEEM/FCID, 2018; US-EPA, 2014). This model is commonly used by the U.S. EPA Office of Prevention, Pesticides and Toxic Substances to estimate human dietary exposure.

The DEEM™ - FCID model is designed to perform mean annual (chronic) and 95th percentile daily (acute) exposure analyses for the U.S. population and a wide range of sub-populations based on 2-day food consumption data from the National Health and Nutrition Examination Survey (NHANES; CDC, 2019) What We Eat in America (WWEIA) 2005-2010 (USDA-NAL, 2019). The 'foods-as-eaten' data in NHANES were converted to raw agricultural commodities and other basic ingredients based on the EPA/USDA FCID recipe set as of August 2014 (DEEM/FCID, 2018).

While the consumption and recipe data that form the basis of the DEEM™ - FCID model are derived from a U.S. survey, the data are applicable to other populations with similar dietary consumption patterns such as Canada, New Zealand, Japan and Italy (WHO-GEMS, 2019). In addition, dietary consumption patterns for specific sub-populations, for example Hispanics or Asians, are likely similar in the U.S. and other countries.

Conservative total replacement scenarios were utilized for both acute and chronic exposures, assuming that maize in each foodstuff was derived from DP23211 maize grain. It was assumed that no degradation of proteins occurred during processing or cooking of corn flour, corn-flour-baby food, corn meal, corn meal-baby food and corn bran foodstuff categories. However, in the case of corn oil, corn starch, and corn syrup foodstuff categories, protein contents are considered to be zero due to processing (CRA, 2006a; CRA, 2006b; CRA, 2006c; Hefle and Taylor, 1999); therefore, consumption of these foodstuffs was not considered for exposure estimates.

VII-B.3.e.2. IPD072Aa Protein Human Exposure Assessment Results

Mean annual (chronic) exposure was highest for the 'children ages 3-5 years' subgroup with an exposure of 0.001160 mg/kg BW/day for the IPD072Aa protein.

The highest 95th percentile per capita daily (acute) exposure for the IPD072Aa protein was in the 'children ages 3-5 years' subgroup with an exposure of 0.004775 mg/kg BW/day. The highest 95th percentile users daily (acute) exposure for the IPD072Aa protein was in the 'children ages 1-2 years' subgroup with an exposure of 0.006237 mg/kg BW/day.

The actual exposure to the expressed IPD072Aa protein from DP23211 maize grain in the diet is expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus grain containing the IPD072Aa protein will be mixed with other grain potentially not containing the IPD072Aa protein, and (2) reductions in concentrations will likely occur during processing to produce maize flour and other processed commodities.

VII-B.3.e.3. IPD072Aa Protein Human Exposure Assessment Conclusions

Dietary risk was assessed for the IPD072Aa protein by calculating the margin of exposure (MOE) by dividing the respective protein dose administered in the IPD072Aa protein 14-day acute toxicity study where no adverse effects were observed (2000 mg/kg BW (Section VII-B.3.d.8. Evaluation of the Acute Toxicity of IPD072Aa Protein) by the highest respective acute dietary exposure estimate. The 95th percentile per capita daily (acute) exposure MOE calculated using the 'children ages 3-5 years' sub-group value was 431,183, and the 95th percentile users daily (acute) exposure MOE calculated using the 'children ages 1-2 years' sub-group value was 320,667for the IPD072 protein.

Estimated exposure to the IPD072Aa protein from consumption of DP23211 maize grain products is low. Estimated exposure to the IPD072Aa protein expressed in DP23211 maize is well below that which was administered in an acute oral toxicity study where no treatment-related adverse effects were observed, resulting in margins of safety. Therefore, consumption of DP23211 maize grain is expected to be as safe as conventional non-GE maize, especially when accounting for blending and processing

See Appendix 8. Materials and Methods for Human Dietary Exposure Evaluation for further details regarding methods used for human dietary exposure.

VII-B.3.f. Livestock Exposure Assessment of IPDAa Protein in DP23211 Maize

VII-B.3.f.1. IPD072Aa Protein Livestock Exposure Assessment Methods

Utilizing the mean concentration of the IPD072Aa protein in grain or forage from DP23211 maize (Table 19), daily dietary exposure (DDE) to the IPD072Aa protein from consumption of DP23211 maize grain and/or forage/silage was calculated for various livestock species using estimates of animal body weight (BW), daily feed intake, and grain and forage/silage inclusion rates specific for North America (OECD, 2013; corn, field). The following conservative total replacement scenarios were utilized:

- 100% DP23211 maize grain replacement for poultry (broiler, layer, turkey), swine (breeding, finishing), cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize forage/silage replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize grain and forage/silage combination replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb)

VII-B.3.f.2. IPD072Aa Protein Livestock Exposure Assessment Results

The highest estimated DDE to the IPD072Aa protein for the 100% DP23211 maize replacement scenarios was observed in Dairy Cattle with consumption of 0.33 mg/kg BW/day.

In practice, the actual livestock dietary exposures to the IPD072Aa protein is expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus maize sources containing DP23211 the IPD072Aa protein will be mixed with other maize grain sources potentially not containing the IPD072Aa protein, and (2) the estimates were highly conservative in their maize grain and/or forage/silage incorporation rates, not accounting for typical blending with other feedstuffs for adequate nutrient levels and least-cost formulations.

The estimated DDE to the IPD072Aa protein, based on conservative assumptions (*e.g.*, all maize grain and/or forage/silage in the diet derived from DP23211 maize) was compared with the dose administered in a corresponding acute oral toxicity study where no treatment-related effects were observed in mice (IPD072Aa, 2000 mg/kg BW) presented in Section VII-B.3.d.8. Evaluation of the Acute Toxicity of IPD072Aa Protein; to determine margin of exposure (MOE). The calculated MOE values indicate margins of safety for the IPD072Aa proteinfrom DP23211 maize grain and/or forage/silage in livestock diets as values ranged from 6,139 to 151,261 across species for all three total replacement scenarios.

VII-B.3.f.3. IPD072Aa Protein Livestock Exposure Assessment Conclusions.

The estimated livestock exposures to the IPD072Aa protein from consumption of DP23211 maize grain and/or forage/silage are low. Estimated exposure to the IPD072Aa protein expressed in DP23211 maize grain and/or forage/silage is well below that which was administered in an acute oral toxicity study where no treatment-related adverse effects were observed, resulting in margins of safety. Therefore, consumption of DP23211 maize grain and/or forage/silage is not expected to pose a risk to livestock, especially when accounting for market share and blending.

See Appendix 9. Materials and Methods for Livestock Dietary Exposure for further details regarding methods used for livestock dietary exposure.

VII-B.4. Conclusions on the Human and Livestock Safety of IPD072Aa Protein in DP23211 Maize

In conclusion, protein characterization results via SDS-PAGE, western blot, peptide mapping, N-terminal amino acid sequence, and glycoprotein analysis have demonstrated that the IPD072Aa protein derived from DP23211 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation. Microbially derived IPD072Aa protein was demonstrated to be equivalent to the DP23211 maize-derived IPD072Aa protein for use in safety testing.

The allergenic potential of the IPD072Aa protein was evaluated by assessing the IPD072Aa protein source organism and history of safe use; a bioinformatic comparison of the amino acid sequence of the IPD072Aa protein with known or putative protein allergen sequences; evaluation of the stability of the IPD072Aa protein using *in vitro* gastric and intestinal digestion models; determination of the IPD072Aa protein glycosylation status; and evaluation of the heat lability of the IPD072Aa protein using a sensitive insect bioassay. The toxicity potential of the IPD072Aa protein was evaluated by an acute toxicity study in mice and a bioinformatic comparison of the IPD072Aa amino acid sequence to known and putative protein toxins.

The results showed that the IPD072Aa protein is digested in SGF and SIF digestion analyses. The IPD072Aa protein autoclaved for approximately 30 minutes at 121 °C was inactive against WCR when incorporated in an artificial diet. The bioinformatic comparisons of the IPD072Aa protein sequence to known and putative allergen and toxin sequences showed that the IPD072Aa protein is unlikely to be allergenic or toxic for humans or animals. The acute oral toxicity assessment determined the LD_{50} of IPD072Aa protein to be greater than 2000 mg/kg. These data support the conclusion that the IPD072Aa protein in DP23211 maize is as safe as conventional maize for the food and feed supply.

Based on this weight of evidence, consumption of the IPD072Aa protein is unlikely to cause an adverse effect on humans or animals.

VII-C. Characterization of the Human and Livestock Safety of the PAT Protein in DP23211 Maize

The PAT protein has been risk-assessed in previously authorized maize events, and it has been determined to be unlikely to be a potential toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, glycosylation, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize. In this section we present DP23211 maize PAT protein expression data, updated bioinformatics comparisons of the PAT protein, the deduced amino acid sequence, Western blot data, expression data, and human and livestock exposure assessments. Results show that the PAT protein from DP23211 maize shows no

homology to known toxins or allergens, the deduced amino acid sequence, size and immunoreactivity is as expected and equivalent to the PAT protein in previously authorized maize events. Human and livestock exposure to the PAT protein through consumption of DP23211 maize is low and has a history of safe consumption. These results support the conclusion that the PAT protein in DP23211 maize is as safe as conventional maize for the food and feed supply and is unlikely to cause an adverse effect on humans or animals.

VII-C.1. Concentration of PAT Protein in DP23211 Maize

The ranges and standard deviations of PAT protein mean concentrations in leaf, root, and whole-plant tissues over the course of the growing season, as well as the mean concentrations in pollen, forage, and grain, are summarized in Table 28 for DP23211 maize.

Table 28. Across-Site Summary of PAT Protein Concentrations in DP23211 Maize

Tissue	Growth Stage	Mean (ng/mg tissue dw)	Range (ng/mg tissue dw)	Standard Deviation (ng/mg tissue dw)
Root	V6	7.7	2.2 - 11	2.6
	V9	4.5	2.4 - 7.8	1.4
	R1	3.5	1.6 - 5.1	0.93
	R4	1.6	0.66 - 3.9	1.0
	R6	0.65[a]	<0.054 - 2.3	0.65[a]
Leaf	V9	7.6	4.0 - 11	1.9
	R1	7.8	5.3 - 13	1.5
	R4	3.6	2.3 - 6.6	1.2
	R6	<0.11	<0.11	ND
Pollen	R1	58	47 - 85	13
Whole Plant	R1	9.2	6.2 - 14	2.0
	R6	1.1[a]	<0.036 - 4.0	1.2[a]
Forage	R4	8.2	4.8 - 11	1.7
Grain	R6	5.1	2.5 - 8.1	1.6

Note: Growth stages (Abendroth et al., 2011). Lower limit of quantification (LLOQ) in ng/mg tissue dry weight. Not determined (ND); all samples were below the LLOQ.

[a] Some sample results were below the LLOQ (4 of 24 for root R6, and 2 of 24 for whole plant R6). A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.

VII-C.2. Amino Acid Sequence of the PAT Protein

The gene encoding the PAT protein in DP23211 maize, referred to as the *mo-pat* gene, was isolated from *Streptomyces viridochromogenes* with codon-optimization for expression in maize. The deduced amino acid sequence from the translation of the *mo-pat* gene is identical to the deduced amino acid sequence from the translation of the *pat* gene. The PAT protein encoded by the *pat* and *mo-pat* genes is 183 amino acids in length and has a molecular weight of approximately 21 kDa (Figure 38).

```
PAT(pat)       1 MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL
PAT(mo-pat)    1 MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL

PAT(pat)      51 ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
PAT(mo-pat)   51 ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL

PAT(pat)     101 GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
PAT(mo-pat)  101 GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA

PAT(pat)     151 AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI*
PAT(mo-pat)  151 AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI*
```

Figure 33. Sequence Alignment of the Deduced Amino Acid Sequence of the PAT Protein Encoded by *mo-pat* and *pat* Genes

Deduced amino acid sequence alignment, where PAT (*pat*) represents the deduced amino acid sequence from the translation of the *pat* gene that is found in a number of authorized events across several different crops that are currently in commercial use (Hérouet et al., 2005; USDA-APHIS, 2001; USDA-APHIS, 2005; USDA-APHIS, 2013). The PAT (*mo-pat*) sequence in Figure 38 represents the deduced amino acid sequence from translation of the *mo-pat* gene. The asterisk (*) indicates the translational stop codon

VII-C.3. PAT Protein Function and Activity

The *mo-pat* gene expresses the PAT protein that confers tolerance to glufosinate-ammonium, the active ingredient in phosphinothricin herbicides. This protein is identical to the protein found in a number of authorized events that are currently in commercial use. Maize containing the PAT protein has been commercially grown in the United States since 1996. PAT protein safety has been reviewed and authorized for food and feed use by regulatory authorities in 20 different countries and/or regions. Authorizations for GE plants that express the PAT protein have been issued in 7 species of plants and total over 450 authorized uses (ILSI, 2016).

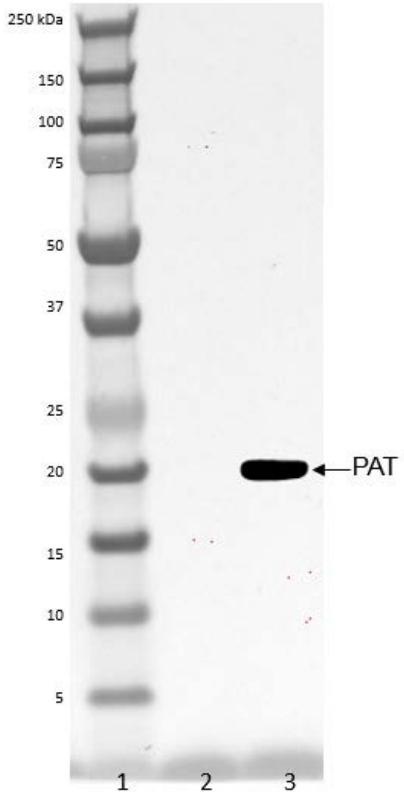
The mode of action of the PAT protein has been previously characterized and described (CERA, 2011b; Hérouet *et al.*, 2005). The PAT protein confers tolerance to glufosinate-ammonium, the active ingredient in phosphinothricin herbicides. Glufosinate chemically resembles the amino acid glutamate and acts to inhibit an enzyme, called glutamine synthetase, which is involved in the synthesis of glutamine. Glutamine synthetase is also involved in ammonia detoxification. Due to its similarity to glutamate, glufosinate blocks the activity of glutamine

synthetase, resulting in reduced glutamine levels and a corresponding increase in concentrations of ammonia in plant tissues, leading to cell membrane disruption and cessation of photosynthesis resulting in plant death. The PAT protein confers tolerance to glufosinate-ammonium herbicides by acetylating phosphinothricin, an isomer of glufosinate-ammonium, thus detoxifying the herbicide (CERA, 2011b; Hérouet *et al.*, 2005).

VII-C.4. Characterization and Equivalence of the PAT Protein in DP23211 Maize

Western blot analysis demonstrated that the PAT protein was immunoreactive to a PAT monoclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 21 kDa. Results are presented in Figure 39.

Methods used to characterize the PAT protein are presented in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS Sample Buffer Blank
3	DP23211 Maize-Derived PAT Protein

Note: kilodalton (kDa).

[a] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 34. Western Blot Analysis of the DP23211 Maize-Derived PAT Protein

VII-C.5. Allergenicity and Toxicity Assessment of the PAT Protein in DP23211 Maize

DP23211 maize was evaluated by examining the allergenic and toxic potential of the PAT protein. The PAT protein has been risk-assessed in previously authorized maize events, and it has been determined to be unlikely to be a potential allergen or toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize (USDA-APHIS, 2001; USDA-APHIS, 2005; USDA-APHIS, 2013). Updated bioinformatic analyses support the original conclusions that the PAT protein is unlikely to be an allergen or toxin. These data support the conclusion that the PAT protein in DP23211 maize is safe for the food and feed supply.

Bioinformatic Toxicity Evaluation of PAT Protein

Assessing expressed proteins for potential toxicity is a critical part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically modified plant products (Codex Alimentarius Commission, 2003). The potential toxicity of the PAT protein was assessed by comparison of its sequence to the sequences in the internal Corteva Agriscience (internal) toxin database. The internal toxin database is a subset of sequences found in UniProtKB/Swiss-Prot (http://www.uniprot.org/). To produce the internal toxin database, the manually annotated proteins in UniProtKB/Swiss-Prot are filtered for molecular function by keywords that could imply toxicity or adverse health effects (*e.g.*, toxin, hemagglutinin, vasoactive, etc.). The internal toxin database is updated annually. The search between the PAT protein sequence and protein sequences in the internal toxin database was conducted with BLASTP using default parameters, except that low complexity filtering was turned off, the *E*-value threshold was set to 10^{-4}, and unlimited alignments were returned.

No alignments with an E-value ≤ 10^{-4} were returned between the PAT protein sequence and any protein sequence in the internal toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the PAT protein.

Bioinformatic Allergenicity Evaluation of PAT Protein

Assessing expressed proteins for potential cross-reactivity with known or putative allergens is a critical part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically-modified plant products (Codex Alimentarius Commission, 2003). In this study, a bioinformatic assessment of the PAT protein sequence for potential cross-reactivity with known or putative allergens was conducted according to relevant guidelines (Codex Alimentarius Commission, 2003; EFSA, 2010; EFSA Panel on Genetically Modified Organisms (GMO), 2011; FAO/WHO, 2001a).

Two separate searches for the PAT protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2018 database (February 2018) available at http://comparedatabase.org. This peer-reviewed database is a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergenicity Technical Committee (PATC) and is comprised of 2,038 sequences. The first search used the PAT protein sequence as the query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the allergen sequences. The search was conducted using default parameters, except the *E*-score threshold was set to 10^{-4}. An *E*-score threshold of 10^{-4} has been shown to be an appropriate value for allergenicity searches (Mirsky et al., 2013). The generated alignments were examined to identify any that are 80 residues or longer and possess a sequence identity of 35% or greater. The second search used a Perl script developed by Pioneer (runLinearEpitopeScreen.pl) to identify any contiguous 8-residue identical matches between the PAT protein sequence and the allergen sequences.

Results of the search of the PAT protein sequence against the COMPARE database of known and putative allergen sequences found no alignments that were 80 residues or longer with a sequence identity of 35% or greater. No contiguous 8-residue matches between the PAT protein sequence and the allergen sequences were identified in the second search.

VII-C.6. Human Exposure Assessment of the PAT Protein in DP23211 Maize

The intended use(s) and degree of exposure can be considered in assessing the safety of a GE crop. This consideration includes the effect(s) (if any) of the level of the food or food product in the diet, patterns of dietary consumption, and the defining characteristics of populations that consume the crop of interest (OECD, 1993).

The level of expression of the PAT proteins was measured in edible tissues and reported in Table 28. These values were then used to estimate potential exposure to humans and livestock as reported below.

VII-C.6.a. PAT Protein Human Exposure Assessment Methods

Dietary exposure to the PAT protein was calculated utilizing the mean concentration of the PAT protein in DP23211 maize grain presented in Table 28, along with consumption data from the Dietary Exposure Evaluation Model – Food Commodity Intake Database (DEEM™ - FCID), Version 4.02 (DEEM/FCID, 2018; US-EPA, 2014). This model is commonly used by the U.S. EPA Office of Prevention, Pesticides and Toxic Substances to estimate human dietary exposure.

The DEEM™ - FCID model is designed to perform mean annual (chronic) and 95th percentile daily (acute) exposure analyses for the U.S. population and a wide range of sub-populations based on 2-day food consumption data from the National Health and Nutrition Examination Survey (NHANES; CDC, 2019) What We Eat in America (WWEIA) 2005-2010 (USDA-NAL, 2019). The

'foods-as-eaten' data in NHANES were converted to raw agricultural commodities and other basic ingredients based on the EPA/USDA FCID recipe set as of August 2014 (DEEM/FCID, 2018).

While the consumption and recipe data that form the basis of the DEEM™ - FCID model are derived from a U.S. survey, the data are applicable to other populations with similar dietary consumption patterns such as Canada, New Zealand, Japan and Italy (WHO-GEMS, 2019). In addition, dietary consumption patterns for specific sub-populations, for example Hispanics or Asians, are likely similar in the U.S. and other countries.

Conservative total replacement scenarios were utilized for both acute and chronic exposures, assuming that maize in each foodstuff was derived from DP23211 maize grain. It was assumed that no degradation of proteins occurred during processing or cooking of corn flour, corn-flour-baby food, corn meal, corn meal-baby food and corn bran foodstuff categories. However, in the case of corn oil, corn starch, and corn syrup foodstuff categories, protein contents are considered to be zero due to processing (CRA, 2006a; CRA, 2006b; CRA, 2006c; Hefle and Taylor, 1999); therefore, consumption of these foodstuffs was not considered for exposure estimates.

VII-C.6.b. PAT Protein Human Exposure Assessment Results

Mean annual (chronic) exposure was highest for the 'children ages 3-5 years' subgroup with an exposure of 0.002817mg/kg BW/day for the PAT protein.

The highest 95th percentile per capita daily (acute) exposures for the PAT protein was in the 'children ages 3-5 years' subgroup with an exposure of 0.011596 mg/kg BW/day. The highest 95th percentile users daily (acute) exposures for the PAT protein was in the 'children ages 1-2 years' subgroup with an exposure of 0.015146 mg/kg BW/day.

The actual exposure to the PAT protein from DP23211 maize grain in the diet is expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus grain containing the PAT protein will be mixed with other grain potentially not containing the PAT protein, and (2) reductions in concentrations will likely occur during processing to produce maize flour and other processed commodities.

Dietary risk was assessed for the PAT protein by calculating the margin of exposure (MOE) by dividing the respective protein dose administered in a 14-day acute toxicity study where no adverse effects were observed (5000 mg/kg BW (Brooks, 2000)) by the highest respective acute dietary exposure estimate. The 95th percentile per capita daily (acute) exposure MOE calculated using the 'children ages 3-5 years' sub-group value was 431,183 , and the 95th percentile users daily (acute) exposure MOE calculated using the 'children ages 1-2 years' sub-group value was 330,120 for the PAT protein.

VII-C.6.c. PAT Protein Human Exposure Assessment Conclusions

Estimated exposure to the PAT protein from consumption of DP23211 maize grain products is low. Estimated exposure to the PAT protein expressed in DP23211 maize is well below that which was administered in an acute oral toxicity study where no treatment-related adverse effects were observed, resulting in margins of safety. Therefore, consumption of DP23211 maize grain is expected to be as safe as conventional non-GE maize, especially when accounting for blending and processing

See Appendix 8. Materials and Methods for Human Dietary Exposure Evaluation for further details regarding methods used for human dietary exposure.

VII-C.7. Livestock Exposure Assessment of the PAT Protein in DP23211 Maize

VII-C.7.a. PAT Protein Livestock Exposure Assessment Methods

Utilizing the mean concentrations of the PAT protein in grain or forage from DP23211 maize presented in Table 28, daily dietary exposure (DDE) to the PAT protein from consumption of DP23211 maize grain and/or forage/silage were calculated for various livestock species using estimates of animal body weight (BW), daily feed intake, and grain and forage/silage inclusion rates specific for North America (OECD, 2013; corn, field). The following conservative total replacement scenarios were utilized:

- 100% DP23211 maize grain replacement for poultry (broiler, layer, turkey), swine (breeding, finishing), cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize forage/silage replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize grain and forage/silage combination replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb)

VII-C.7.b. PAT Protein Livestock Exposure Assessment Results

The highest estimated DDE to the PAT protein for the 100% DP23211 maize replacement scenarios was observed in Broilers with a value of 0.31 mg/kg BW/day.

In practice, the actual livestock dietary exposure to the PAT protein is expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus maize sources containing DP23211 the PAT protein will be mixed with other maize grain sources potentially not containing the PAT protein, and (2) the estimates were highly conservative in their maize grain and/or forage/silage incorporation rates, not accounting for typical blending with other feedstuffs for adequate nutrient levels and least-cost formulations.

The estimated DDE to the PAT protein, based on conservative assumptions (*e.g.*, all maize grain and/or forage/silage in the diet derived from DP23211 maize) was compared with the dose

administered in a corresponding acute oral toxicity study where no treatment-related effects were observed in mice (PAT, 5000 mg/kg BW (Brooks, 2000) to determine margin of exposure (MOE). The calculated MOE value indicate margins of safety for the PAT protein from DP23211 maize grain and/or forage/silage in livestock diets as values ranged from 16,340 to 223,354 across all species for all three total replacement scenarios.

VII-C.7.c. PAT Protein Livestock Exposure Assessment Conclusions

Estimated exposures to the PAT protein expressed in DP23211 maize grain and/or forage/silage are well below that which was administered in the PAT acute oral toxicity study where no treatment-related adverse effects were observed, resulting in margins of safety. Therefore, consumption of DP23211 maize grain and/or forage/silage is not expected to pose a risk to livestock, especially when accounting for market share and blending.

See Appendix 9. Materials and Methods for Livestock Dietary Exposure for further details regarding methods used for livestock dietary exposure.

VII-C.8. Conclusions on the Human and Livestock Safety of the PAT Protein in DP23211 Maize

The PAT protein has been risk-assessed in previously authorized maize events, and has been determined to be unlikely to be a potential toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, glycosylation, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize (USDA-APHIS, 2001; USDA-APHIS, 2005; USDA-APHIS, 2013). Updated bioinformatics comparisons of the PAT protein show no homology to known toxins or allergens. Western blot analysis demonstrated that the PAT protein derived from DP23211 maize is equivalent in size and immunoreactivity to the PAT protein in previously authorized events. Exposure of the PAT protein through consumption of DP23211 maize is low and is not anticipated to pose a risk to humans or livestock.

Based on this body of evidence, it is not anticipated that the consumption of the PAT protein will cause an adverse effect in humans or animals.

VII-D. Characterization of the Human and Livestock Safety of the PMI Protein in DP23211 Maize

The PMI protein has been risk-assessed in previously authorized maize events, and has been determined to be unlikely to be a potential toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, glycosylation, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize. In this section, we present DP23211 maize PMI protein expression data, updated bioinformatics comparisons of the PMI protein, the deduced amino acid sequence, Western blot data, expression data, and human and livestock exposure assessments. Results show that the PMI protein from DP23211 maize shows no

homology to known toxins or allergens, the deduced amino acid sequence, size and immunoreactivity is as expected and equivalent to previous PMI protein data. Human and livestock exposure to the PMI protein through consumption of DP23211 maize is low and has a history of safe consumption. These results support the conclusion that the PMI protein in DP23211 maize is as safe as conventional maize for the food and feed supply and is unlikely to cause an adverse effect on humans or animals.

VII-D.1 Concentration of PMI Protein in DP23211 Maize

The ranges and standard deviations of PMI protein mean concentrations in leaf, root, and whole-plant tissues over the course of the growing season, as well as the mean concentrations in pollen and grain, are summarized in Table 29 for DP23211 maize.

Table 29. Across-Site Summary of PMI Protein Concentrations in DP23211 Maize

Tissue	Growth Stage	Mean (ng/mg tissue dw)	Range (ng/mg tissue dw)	Standard Deviation (ng/mg tissue dw)
Root	V6	12	5.7 - 21	4.4
	V9	6.5	3.3 - 11	2.2
	R1	5.3	2.7 - 11	1.9
	R4	3.7	2.1 - 5.4	0.96
	R6	2.6[a]	<0.27 - 5.7	1.6[a]
Leaf	V9	11	6.6 - 20	3.4
	R1	12	7.2 - 19	2.7
	R4	29	17 - 43	6.7
	R6	0.30[a]	<0.54 - 0.66	0.097[a]
Pollen	R1	33	28 - 43	4.4
Whole Plant	R1	8.9	7.0 - 12	1.4
	R6	3.6[a]	<1.8 - 8.8	2.2[a]
Forage	R4	9.4	6.2 - 17	2.3
Grain	R6	4.3	2.3 - 6.3	1.1

Note: Growth stages (Abendroth et al., 2011). Lower limit of quantification (LLOQ) in ng/mg tissue dry weight.

[a] Some, but not all, sample results were below the LLOQ (3 of 24 for root R6, 22 of 24 for leaf R6, and 7 of 24 for whole plant R6). A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.

VII-D.2. Amino Acid Sequence of the PMI Protein

The gene encoding the PMI protein in DP23211 maize, referred to as the *pmi* gene, was isolated from *Escherichia coli.* PMI served as a selectable marker during transformation which allowed for tissue growth using mannose as the carbon source. The deduced amino acid sequence from the translation of the PMI is 391 amino acids in length and has a molecular weight of approximately 43 kDa (Figure 35).

```
  1  MQKLINSVQN YAWGSKTALT ELYGMENPSS QPMAELWMGA HPKSSSRVQN
 51  AAGDIVSLRD VIESDKSTLL GEAVAKRFGE LPFLFKVLCA AQPLSIQVHP
101  NKHNSEIGFA KENAAGIPMD AAERNYKDPN HKPELVFALT PFLAMNAFRE
151  FSEIVSLLQP VAGAHPAIAH FLQQPDAERL SELFASLLNM QGEEKSRALA
201  ILKSALDSQQ GEPWQTIRLI SEFYPEDSGL FSPLLLNVVK LNPGEAMFLF
251  AETPHAYLQG VALEVMANSD NVLRAGLTPK YIDIPELVAN VKFEAKPANQ
301  LLTQPVKQGA ELDFPIPVDD FAFSLHDLSD KETTISQQSA AILFCVEGDA
351  TLWKGSQQLQ LKPGESAFIA ANESPVTVKG HGRLARVYNK L*
```

Figure 35. **Deduced Amino Acid Sequence of the PMI Protein**
The deduced amino acid sequence from the translation of the *pmi* gene from plasmid PHP74643. The asterisk (*) indicates the translational stop codon. The full-length protein is 391 amino acids in length and has a molecular weight of approximately 43 kDa.

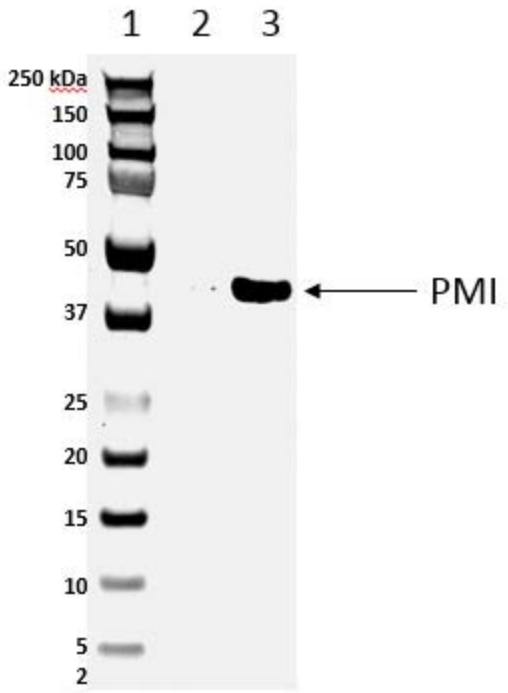
VII-D.3. PMI Protein Function and Activity

The mode of action of PMI has been previously characterized and described (Negrotto et al., 2000; Privalle, 2002; Reed et al., 2001; Weisser et al., 1996). PMI is widely present in nature and is expressed in fungi, insects, plants, and mammals (Slein, 1950; US-EPA, 2004). The United States EPA has granted an exemption from the requirement of a tolerance for the PMI protein as an inert ingredient in plants (US-EPA, 2004). The PMI protein catalyzes the reversible interconversion between mannose-6-phosphate and fructose-6-phosphate. Mannose is phosphorylated by hexokinase to mannose-6-phosphate and in the presence of PMI enters the glycolytic pathway after isomerization to fructose 6-phosphate. In the absence of PMI, mannose-6-phosphate accumulates in the plant cells and inhibits glycolysis; additionally, high levels of mannose can lead to other impacts on photosynthesis and ATP production (Negrotto et al., 2000; Privalle, 2002). However, in the presence of PMI, plant cells may survive on media containing mannose as a carbon source, thus allowing PMI to be utilized as a selectable marker (Negrotto et al., 2000; Reed et al., 2001).

VII-D.4. Characterization and Equivalence of the PMI Protein in DP23211 Maize

Western blot analysis demonstrated that the PMI protein was immunoreactive to a PMI monoclonal antibody and visible as a band consistent with the expected molecular weight of approximately 43 kDa (Figure 36).

Methods used to characterize the PMI protein are presented in Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS Sample Buffer Blank
3	DP23211 Maize-Derived PMI Protein

Note: kilodalton (kDa).

[a] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 36. Western Blot Analysis of the DP23211 Maize-Derived PMI Protein

VII-D.5. Allergenicity and Toxicity Assessment of the PMI Protein in DP23211 Maize

DP23211 maize was evaluated by examining the allergenic and toxic potential of the PMI protein. The PMI protein has been risk-assessed in previously authorized maize events, and it has been determined to be unlikely to be a potential allergen or toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, glycosylation, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize (USDA-APHIS, 2011). Updated bioinformatic analyses support the original conclusions that the PMI protein is unlikely to be an allergen or toxin. These data support the conclusion that the PMI protein in DP23211 maize is safe for the food and feed supply.

Bioinformatic Toxicity Evaluation of the PMI Protein

The NCBI Entrez® Protein Database search identified 1000 sequences with potentially significant similarity to the PMI amino acid sequence (*i.e., E*-values less than 1×10^{-5}). All 1000 of these sequences were grouped into categories and then grouped by the source organism for each set of sequences.

Of the 1000 sequences, all were identified as PMI or related proteins. The *E*-values for alignments between these sequences and the PMI amino acid sequence were all 0, indicating that all aligning sequences were also PMI proteins.

There were no alignments between the PMI amino acid sequence and any proteins in the Syngenta toxin database with significant sequence similarity (*E*-value < 1×10^{-5}). Five alignments below the upper reportable *E*-value (*E*-value < 10) were observed. The most similar alignment with a protein from the database had an *E*-value of 2.07901. This *E*-value is greater than the significance threshold *E*-value of 1×10^{-5} and indicates that the alignment is unlikely to be of biological relevance.

An assessment of the PMI amino acid sequence using a comprehensive similarity search of a non-redundant NCBI Entrez® Protein Database and a toxin-specific database created from the NCBI Entrez® Protein listing (2019) supports the conclusion that the PMI amino acid sequence shows no biologically relevant similarity to any known or putative toxins.

Bioinformatic Allergenicity Evaluation of the PMI Protein

To determine whether the PMI amino acid sequence showed biologically relevant similarity to amino acid sequences of known or putative allergens, two different searches were performed against the Comprehensive Protein Allergen Resource (COMPARE) database, version 2019, which contains 2081 amino acid sequences of known and putative allergens. A full-length sequence search using FASTA, and a separate search for exact matches of eight or more contiguous amino acids, were used to compare the PMI protein to each of the known or putative allergen

sequences. In the FASTA search, no sequence similarity greater than 35% shared identity over 80 or more amino acids was observed between the PMI amino acid sequence and any entry in the COMPARE database.

Safety evaluations of PMI have been previously disclosed by the developer (Syngenta Biotechnology, Inc.) (USDA-APHIS, 2001; USDA-APHIS, 2005; Vlachos and Huber, 2011; Ward and Huber, 2007)

In the eight-amino acid match search, a single match between the PMI protein and α-parvalbumin from *Rana* species CH2001 was observed. This alignment has been previously reported in assessments of the sequence similarity of the PMI protein to known and putative allergens (first reported in Rabe 2004). Further investigation using serum IgE screening demonstrated no cross-reactivity between the PMI protein and the α-parvalbumin protein using serum from the single individual known to have demonstrated IgE-mediated allergy to this specific α-parvalbumin from *Rana* species CH2001. The results indicated that the allergic patient's serum IgE does not recognize any portion of the PMI protein as an allergenic epitope. The present bioinformatics study reassessed the similarity of the PMI protein to known allergens using the 2019 COMPARE allergen database.

Together, these results support the conclusion that the PMI protein shares no biologically relevant amino acid sequence similarity to known or putative protein allergens.

VII-D.6. Human Exposure Assessment of the PMI Protein in DP23211 Maize

The intended use(s) and degree of exposure can be considered in assessing the safety of a GE crop. This consideration includes the effect(s) (if any) of the level of the food or food product in the diet, patterns of dietary consumption, and the defining characteristics of populations that consume the crop of interest (OECD, 1993).

The levels of expression of the PMI protein was measured in edible tissues and is reported in Table 29. These values were then used to estimate potential exposure to humans and livestock as reported below.

VII-D.6.a. PMI Protein Human Exposure Assessment Methods

Dietary exposure to the PMI protein was calculated utilizing the mean concentration of PMI protein in DP23211 maize grain presented in Table 29, along with consumption data from the Dietary Exposure Evaluation Model – Food Commodity Intake Database (DEEM™ - FCID), Version 4.02 (DEEM/FCID, 2018; US-EPA, 2014). This model is commonly used by the U.S. EPA Office of Prevention, Pesticides and Toxic Substances to estimate human dietary exposure.

The DEEM™ - FCID model is designed to perform mean annual (chronic) and 95th percentile daily (acute) exposure analyses for the U.S. population and a wide range of sub-populations based on

2-day food consumption data from the National Health and Nutrition Examination Survey (NHANES; CDC, 2019) What We Eat in America (WWEIA) 2005-2010 (USDA-NAL, 2019). The 'foods-as-eaten' data in NHANES were converted to raw agricultural commodities and other basic ingredients based on the EPA/USDA FCID recipe set as of August 2014 (DEEM/FCID, 2018).

While the consumption and recipe data that form the basis of the DEEM™ - FCID model are derived from a U.S. survey, the data are applicable to other populations with similar dietary consumption patterns such as Canada, New Zealand, Japan and Italy (WHO-GEMS, 2019). In addition, dietary consumption patterns for specific sub-populations, for example Hispanics or Asians, are likely similar in the U.S. and other countries.

Conservative total replacement scenarios were utilized for both acute and chronic exposures, assuming that maize in each foodstuff was derived from DP23211 maize grain. It was assumed that no degradation of proteins occurred during processing or cooking of corn flour, corn-flour-baby food, corn meal, corn meal-baby food and corn bran foodstuff categories. However, in the case of corn oil, corn starch, and corn syrup foodstuff categories, protein contents are considered to be zero due to processing (CRA, 2006a; CRA, 2006b; CRA, 2006c; Hefle and Taylor, 1999); therefore, consumption of these foodstuffs was not considered for exposure estimates.

VII-D.6.b. PMI Protein Human Exposure Assessment Results

The mean annual (chronic) exposure was highest for the 'children ages 3-5 years' subgroup with an exposure of 0.002375 mg/kg BW/day for the PMI protein.

The highest 95th percentile per capita daily (acute) exposure for the PMI protein was in the 'children ages 3-5 years' subgroup with an exposure of 0.009777 mg/kg BW/day. The highest 95th percentile users daily (acute) exposures for the PMI protein was in the 'children ages 1-2 years' subgroup with an exposure of 0.012770 mg/kg BW/day.

The actual exposure to the PMI protein from DP23211 maize grain in the diet is expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus grain containing the PMI protein will be mixed with other grain potentially not containing the PMI protein, and (2) reductions in concentrations will likely occur during processing to produce maize flour and other processed commodities.

Dietary risk was assessed for the PMI protein by calculating the margin of exposure (MOE) by dividing the respective protein dose administered in a 14-day acute toxicity study where no adverse effects were observed (2000 mg/kg BW (Korgaonkar, 2009) for the PMI protein) by the highest respective acute dietary exposure estimate. The 95th percentile per capita daily (acute) exposure MOEcalculated using the 'children ages 3-5 years' sub-group value was 204,562 , and the 95th percentile users daily (acute) exposure MOE calculated using the 'children ages 1-2 years' sub-group value was 156,617 for the PMIprotein.

VII-D.6.c. PMI Protein Human Exposure Assessment Conclusions

Estimated exposure to the PMI protein from consumption of DP23211 maize grain products is low. Estimated exposure to the PMI protein expressed in DP23211 maize are well below that which was administered in the PMI acute oral toxicity study where no treatment-related adverse effects were observed, resulting in margins of safety. Therefore, consumption of DP23211 maize grain is expected to be as safe as conventional non-GE maize, especially when accounting for blending and processing

See Appendix 8. Materials and Methods for Human Dietary Exposure Evaluation for further details regarding methods used for human dietary exposure.

VII-D.7. Livestock Exposure Assessment of the PMI Protein in DP23211 Maize

VII-D.7.a. PMI Protein Livestock Exposure Assessment Methods

Utilizing the mean concentrations of the PMI protein in grain or forage from DP23211 maize presented in Table 29, daily dietary exposure (DDE) to the PMI protein from consumption of DP23211 maize grain and/or forage/silage were calculated for various livestock species using estimates of animal body weight (BW), daily feed intake, and grain and forage/silage inclusion rates specific for North America (OECD, 2013; corn, field). The following conservative total replacement scenarios were utilized:

- 100% DP23211 maize grain replacement for poultry (broiler, layer, turkey), swine (breeding, finishing), cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize forage/silage replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize grain and forage/silage combination replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb)

VII-D.7.b. PMI Protein Livestock Exposure Assessment Results

The highest estimated DDE to the PMI protein for the 100% DP23211 maize replacement scenarios was observed in Broilers with a value of 0.26 mg/kg BW/day.

In practice, the actual livestock dietary exposures to the PMI protein are expected to be lower than these estimates because (1) maize grain is a highly blended commodity, thus maize sources containing DP23211 maize PMI protein will be mixed with other maize grain sources potentially not containing the PMI protein, and (2) the estimates were highly conservative in their maize grain and/or forage/silage incorporation rates, not accounting for typical blending with other feedstuffs for adequate nutrient levels and least-cost formulations.

The estimated DDEs to the PMI protein, based on conservative assumptions (*e.g.*, all maize grain and/or forage/silage in the diet derived from DP23211 maize) were compared with the dose

administered in a corresponding acute oral toxicity study where no treatment-related effects were observed in mice (PMI, 2000 mg/kg BW (Korgaonkar, 2009)) to determine margin of exposure (MOE). The calculated MOE values indicate margins of safety for the PMI protein from DP23211 maize grain and/or forage/silage in livestock diets as values ranged from 7,752 to 77,936 across all species for all three total replacement scenarios.

VII-D.7.c. PMI Protein Livestock Exposure Assessment Conclusions

The estimated livestock exposure to the PMI protein from consumption of DP23211 maize grain and/or forage/silage is low. Estimated exposure to the PMI protein expressed in DP23211 maize grain and/or forage/silage are well below that which was administered in the PMI acute oral toxicity study where no treatment-related adverse effects were observed, resulting in margins of safety. Therefore, consumption of DP23211 maize grain and/or forage/silage is not expected to pose a risk to livestock, especially when accounting for market share and blending.

See Appendix 9. Materials and Methods for Livestock Dietary Exposure for further details regarding methods used for livestock dietary exposure.

VII-D.8. Conclusions on the Human and Livestock Safety of the PMI Protein in DP23211 Maize

The PMI protein has been risk-assessed in previously authorized maize events, and has been determined to be unlikely to be a potential toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, glycosylation, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize (USDA-APHIS, 2011). Updated bioinformatics comparisons of the PMI protein show no homology to known toxins or allergens. Western blot analysis demonstrated that the PMI protein derived from DP23211 maize is equivalent in size and immunoreactivity. Exposure of the PMI protein through consumption of DP23211 maize is low and is not anticipated to pose a risk to humans or livestock.

Based on this body of evidence, it is not anticipated that the consumption of the PMI protein will cause an adverse effect in humans or animals.

VII-E. Overall Conclusions of the Safety of the DvSSJ1 dsRNA and the IPD072Aa, PAT, and PMI Proteins Contained in DP23211 Maize

Nucleic acids, including dsRNA, are normal components of human and animal diets, and have a history of safe consumption in food and feed. Conservative estimates demonstrate low potential exposures to DvSSJ1 dsRNA for humans in food and animals in feed and the well-characterized physical, enzymatic, biochemical and molecular barriers to exposure of ingested small RNAs will further reduce potential exposure to DvSSJ1 dsRNA consumed in food and feed from DP23211

Protein characterization results via SDS-PAGE, western blot, peptide mapping, N-terminal amino acid sequence, and glycoprotein analysis have demonstrated that the IPD072Aa protein derived from DP23211 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation. The IPD072Aa protein is digested in SGF and SIF digestion analyses. Heat-treated IPD072Aa protein is inactive against WCR when incorporated in an artificial diet. The bioinformatic comparisons of the IPD072Aa protein sequence to known and putative allergen and toxin sequences showed that the IPD072Aa protein is unlikely to be allergenic or toxic for humans or animals. These data support the conclusion that the IPD072Aa protein in DP23211 maize is as safe as conventional maize for the food and feed supply.

The PAT protein has been risk-assessed in previously authorized maize events, and it has been determined to be unlikely to be a potential toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, glycosylation, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize. Updated bioinformatics comparisons of the PAT protein show no homology to known toxins or allergens. Western blot analysis demonstrated that the PAT protein derived from DP23211 maize is equivalent in size and immunoreactivity. Exposure of the PAT protein through consumption of DP23211 maize is low and is not anticipated to pose a risk to humans or livestock.

The PMI protein has been risk-assessed in previously authorized maize events, and it has been determined to be unlikely to be a potential toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, glycosylation, and acute protein toxicity studies and are relevant for the assessment of DP23211 maize. Updated bioinformatics comparisons of the PMI protein show no homology to known toxins or allergens. Western blot analysis demonstrated that the PMI protein derived from DP23211 maize is equivalent in size and immunoreactivity. Exposure of the PMI protein through consumption of DP23211 maize is low and is not anticipated to pose a risk to humans or livestock.

Based on this weight of evidence, consumption of DvSSJ1 dsRNA and the IPD072Aa, PAT, and PMI proteins in DP23211 maize is unlikely to cause an adverse effect in humans or animals.

VIII. Agronomic Performance Assessment and Ecological Observations

Agronomic and ecological evaluations were conducted to assess the comparability of DP23211 maize to conventional maize. These evaluations form the basis to determine whether DP23211 maize is comparable to conventional maize and is therefore no more likely to pose a plant pest risk.

Agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of maize. To evaluate the agronomic characteristics of DP23211 maize, data were collected on representative characteristics that influence reproduction, crop survival, and potential weediness. In each of these assessments, DP23211 maize was compared to a non-GE, near-isoline control maize that was >95% genetically similar to DP23211 maize but did not carry any recombinant DNA, and, in some experiments, was compared to non-genetically engineered conventional maize lines selected from current Pioneer conventional maize products. In each experiment, DP23211 maize was comparable to the non-GE, near-isoline control maize or conventional comparators, with the exception of the days to flowering and final population measurements. The mean values for days to flowering and final population are within the reference range for each measurement which indicates that these differences are likely not biologically meaningful (Anderson *et al.*, 2020).

The ecological evaluations included observed responses to biotic and abiotic stressors during multi-year and multi-site field trials. These observations were made on DP23211 maize and control maize and tracked the presence of insect and disease stressors in the field and the plant responses. In each case, DP23211 maize responded similarly to the non-GE, near-isoline control maize plants in these trials.

Based on the analyses described below, DP23211 maize is comparable to non-GE conventional maize and would not pose a greater plant pest risk or increased weed potential than non-GE conventional maize.

VIII-A. Germination and Viability Evaluations

To evaluate germination and viability, seeds from the F1 (PHEJW/PHR03) generation (Figure 9 and Table 3) of DP23211 maize were tested for germination assays under warm, cold, and diurnal conditions. The F1 (PHEJW/PHR03) generation of seed was used as F1 (PHEJW/PHR03) hybrid seed is representative of seed that growers would plant in commercial maize fields. A non-GE near-isoline control was used for comparison. In addition, six non-GE conventional maize lines, (P0604 maize, P0760 maize, P0928 maize, P0993 maize, P1151 maize, and P1197 maize), were evaluated in the study to establish a reference range for germination and viability evaluations

but were not included in the statistical analysis. This reference range provided context for any statistical differences observed in the comparisons; if the values for DP23211 maize fell within this reference range, it indicated that DP23211 maize was comparable to conventional maize lines.

Each germination test contained eight replicates of 50 seeds each of DP23211 maize, near-isoline control, and six conventional lines. The "International Rules for Seed Testing 2017", published by the International Seed Testing Association, were used as guidelines for the germination methods and interpretation of results (ISTA, 2017). Each replicate was placed between sheets of moist germination paper and rolled up with a piece of wax paper wrapped around the moist paper, and placed in a growth chamber set to the appropriate test conditions. Evaluations were taken at the end of each germination test, and the number of normal and abnormal germinated seed as well as the number of hard, fresh, or dead ungerminated seed in each roll were counted. Germination rates were reported as a percentage of germinating seed as follows: (number of germinated seeds/total seeds planted) *100. The results are presented in Table 30, Table 31, and Table 32.

For evaluation of viability, germinated seed were considered viable and ungerminated seed classified as dead were considered non-viable. If ungerminated seed classified as hard or fresh had been identified, a tetrazolium chloride (TZ) test would have been conducted to assess viability; however, no hard or fresh seed were identified.

Germination rates in DP23211 maize under warm, cold, and diurnal growing conditions were comparable to those of control maize under corresponding growing conditions.

The data provided here support the conclusion that DP23211 maize is comparable to conventional maize with respect to germination and viability.

Materials and methods used to evaluate germination and viability of DP23211 maize are presented in Appendix 11. Materials and Methods for Agronomic Performance Assessment.

Table 30. Summary of Warm Germination Test Results for DP23211 Maize

Reported Statistic	DP23211 Maize	Control Maize	Reference Range
Frequency[a]	397/400	398/400	92.0% - 100%
Mean[b]	99.3%	99.5%	
Range[b]	98.0% - 100%	98.0% - 100%	
P-Value[c]	1.0000	--	

[a] Total germination frequency across replicates.
[b] Mean and range of germination rates for individual replicates.
[c] P-Value was determined using Fisher's exact test for germination rates.

Table 31. Summary of Cold Germination Test Results for DP23211 Maize

Reported Statistic	DP23211 Maize	Control Maize	Reference Range
Frequency[a]	397/400	397/400	94.0% - 100%
Mean[b]	99.3%	99.3%	
Range[b]	98.0% - 100%	98.0% - 100%	
P-Value[c]	1.0000	--	

[a] Total germination frequency across replicates.
[b] Mean and range of germination rates for individual replicates.
[c] P-Value was determined using Fisher's exact test for germination rates.

Table 32. Summary of Diurnal Germination Test Results for DP23211 Maize

Reported Statistic	DP23211 Maize	Control Maize	Reference Range
Frequency[a]	398/400	397/400	96.0% - 100%
Mean[b]	99.5%	99.3%	
Range[b]	98.0% - 100%	96.0% - 100%	
P-Value[c]	1.0000	--	

[a] Total germination frequency across replicates.
[b] Mean and range of germination rates for individual replicates.
[c] P-Value was determined using Fisher's exact test for germination rates.

VIII-B. Field Trial Evaluations

VIII-B.1. Agronomic Evaluation of DP23211 Maize

Agronomic data were collected from the F1 (PHEJW/PHR03) generation of DP23211 maize and concurrently grown non-GE, near isoline maize (referred to as control maize) during the 2018 growing season at 12 sites in maize-growing regions of the United States (one site in Indiana, Minnesota, Nebraska, and Pennsylvania; two sites in Illinois and Texas; and three sites in Iowa) and Canada (one site in Ontario). More information regarding field trial locations is provided in Figure 37. The F1 generation of seed was used as F1 hybrid seed is representative of seed that

growers would plant in production maize fields. The trial locations provided a range of environmental and agronomic conditions representative of the major maize growing regions of the United States and Canada, where production of DP23211 maize is expected.

Agronomic characteristics of DP23211 maize were evaluated in comparison to concurrently grown non-GE, near-isoline maize (referred to as control maize) to identify statistical differences, and subsequently were evaluated in the context of normal ranges of variation established from concurrently grown non-GE, conventional maize (referred to as reference maize) data.

Evaluation of agronomic characteristics of DP23211 maize included early stand count, days to flowering, pollen viability (shape and color at 0, 30, 60, and 120 minutes), plant height, days to maturity, lodging, final population, dropped ears, yield, harvest grain moisture, and 100-kernel weight (Table 33). Additionally, biotic and abiotic observations were taken by evaluating insect damage incidence, plant pathogen incidence, and abiotic stress at each site throughout the growing season.

Each field trial site was managed to maintain an environment that would produce a successful crop including insect, weed, fertility and irrigation management as needed. Maintenance practices were uniform across all entries in each site, thus agronomic characteristic evaluations comparing DP23211 maize to conventional maize are appropriate. Pest management and maintenance practices are described in further detail in Appendix 11. Materials and Methods for Agronomic Performance Assessment

A randomized complete block design with four blocks was utilized at each site. Each block included DP23211 maize, non-genetically engineered (non-GE) near-isoline control maize (referred to as control maize), and four of the following non-GE conventional maize lines: P0604, 2R602, 35A52, P0760, BK5883, XL5939, P0928, P0993, XL5828, BK6076, XL6158, P1105, P1151, and P1197 (referred to as reference maize). These conventional products were chosen to represent a range of non-genetically engineered hybrids that are planted commercially. Agronomic data collected from the reference maize were used to help determine the normal range of variation for the agronomic characteristics in conventional maize.

Statistical analysis was conducted to compare the agronomic endpoints from DP23211 maize and control maize using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA). Agronomic endpoints were analyzed with linear mixed models, with maize line as a fixed effect and site and the interaction between maize line and site as random effects. Means were estimated for each maize line and compared to test whether there was a significant difference (raw p-value < 0.05) between the means. The approximate degrees of freedom for the statistical test were derived using the Kenward-Roger method (Kenward and Roger, 2009). For each agronomic endpoint, goodness-of-fit of the model was assessed in terms of meeting distributional assumptions of

normally, independently distributed errors with homogeneous variance. Deviations from assumptions were addressed using an appropriate transformation or by fitting heterogeneous error variances across sites. The false discovery rate (FDR) method (Benjamini and Hochberg, 1995; Westfall et al., 1999) was used to control for false positive outcomes across all agronomic endpoints analyzed using linear mixed models, and the adjusted p-value is reported for agronomic endpoints with a raw p-value < 0.05. In cases when a raw P-value indicated a significant difference but the FDR adjusted P-value was > 0.05, it was concluded that the difference was likely a false positive, as described previously (Anderson et al., 2019). For a given agronomic characteristic, when a statistically significant difference (P-value < 0.05) was identified in the across-site analysis, the respective range of individual values from DP23211 maize was compared to the in-study reference range comprised of all individual values across-sites from all non-GM reference maize lines grown in this study.

No statistically significant differences were observed between DP23211 maize and the control maize for 11 of the endpoints that went through across-site analysis via either mixed model analysis or CMH test. A statistically significant difference, before FDR-adjustment, was observed in the across-sites analysis between DP23211 maize and the control maize for the remaining four endpoints: early stand, days to flowering, final population, and yield. After FDR adjustment of p-values, the FDR-adjusted p-value for yield was not significant, indicating that the observed difference was likely a false positive. All individual values for early stand and yield were within the in-study reference range, indicating DP23211 maize is within the range of normal variation for these analytes and the statistical differences are not biologically meaningful. For days to flowering, 39 of 47 values (with 8 values above the upper reference range) for DP23211 maize were within the reference data range. For final population, 46 of 47 values for DP23211 maize were within the reference data range (with 1 value below the lower reference range). The mean values for days to flowering and final population are within the reference range for each measurement which indicates that these differences are likely not biologically meaningful. Mean days to flowering was 59.3 d for control maize and 60.4 d for DP23211 maize and the range for non-GM commercial maize was 51-65 d. Mean final population was 5.9 count/m^2 for control maize and 5.7 count/m^2 for DP23211 maize and the range for non-GM commercial maize ranged from 4.1-6.6 count/m^2. Across sites, results showed a consistent trend between control maize and DP23211 maize, either at parity or slightly later flowering or slightly lower final population. While the difference between DP23211 maize and control maize was statistically different for days to flowering and final population, the slight differences are not considered biologically relevant. The results for these agronomic endpoints are similar to those obtained from commercially available non-modified maize seed lots. The agronomic assessment does not indicate that DP23211 would have an adverse effect on the environment (i.e., enhanced survivability or invasiveness potential) as a result of cultivation.

The results obtained in this evaluation demonstrated that agronomic characteristics of DP23211 maize were comparable to those of conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize. Statistically significant differences were identified in days to flowering and final population, with 39 of 47 observations for days to flowering and 46 of 47 observations for final population within the reference range (Table 34). These differences are not anticipated to be biologically meaningful as they are within the reference range for each measurement. Additionally, the differences observed for days to flowering and final stand count are unlikely to result in DP23211 maize plants with increased weediness potential or survivability, compared to conventional maize which is not considered a weedy or invasive plant (CFIA, 1994; OECD, 2003).

Details of the methods used are presented in Appendix 11. Materials and Methods for Agronomic Performance Assessment.

Figure 37. **Field Site Locations Used for Agronomic Evaluation of DP23211 Maize**

Reference	State/ Province	Soil Texture
1	Iowa	Silty Clay Loam
2	Iowa	Loam
3	Iowa	Silty Clay Loam
4	Illinois	Silt Loam
5	Illinois	Silt Loam
6	Indiana	Silty Clay Loam/Silt Loam/Loam
7	Minnesota	Sandy Clay Loam/Sandy Loam
8	Nebraska	Silty Clay Loam
9	Ontario	Sandy Loam
10	Pennsylvania	Loam
11	Texas	Clay
12	Texas	Clay Loam

Table 33. Agronomic Characteristics Measured

Characteristic	Evaluation Timing[a]	Description	Scale
Early Stand Count	V2-V4	Total number of plants emerged per plot	Numerical count per meter squared
Days to Flowering	Approximately 50% of plants shedding pollen	From the time of planting until approximately 50% of plants have tassels shedding pollen	Days
Plant Height	R4	Height from soil surface to collar of flag leaf for five individual plants	Centimeters
Lodging	R6	Combined score of stalk lodging (number of plants in each plot with stalks broken below the primary ear) and root lodging (number of plants in each plot with stalks leaning approximately 45 degrees or more)	Percentage
Final Population	R6	Total number of remaining plants per plot	Numerical count per meter squared
Days to Maturity	Physiological maturity	Number of days for majority of plants to first reach physiological maturity	Days
Pollen Viability[b]	During active pollen shed	Shape and color at 0, 30, 60, and 120 minutes	Percent of grains with collapsed walls and percent of grains with yellow color
Dropped Ears	R6	Number of dropped ears (ears laying on the ground within each plot)	Numerical count
Harvest Grain Moisture	Approximately R6	Moisture content of harvested grain	Percentage
Yield	Approximately R6	Harvest weight per area adjusted to 15.5% moisture	Calculated bushels per acre
100 Kernel Weight	R6	Total weight of 100 kernels of pooled grain, adjusted to 15.5% moisture	Weight in grams

[a] Refer to Abendroth et al. (2011) for a description of maize growth stages.
[b] Pollen viability has been correlated to pollen shape and color (Luna et al., 2001).

Table 34. Across-Site Analysis of Agronomic Characteristics Results

Agronomic Characteristic	Reported Statistics	Control Maize	DP23211 Maize	Reference Data Range
Early Stand (count/m^2)	Mean	6.1	5.9	3.8 - 6.7
	Range	4.3 - 6.6	4.7 - 6.5	
	Confidence Interval	5.8 - 6.3	5.6 - 6.2	
	Adjusted P-Value	--	0.000566[†]	
	P-Value	--	<0.0001[*]	
Days to Flowering	Mean	59.3	60.4	51 - 65
	Range	53 - 67	53 - 67	
	Confidence Interval	56.9 - 61.7	57.9 - 62.8	
	Adjusted P-Value	--	<0.0001[†]	
	P-Value	--	<0.0001[*]	
Pollen Viability-Shape, 0 minutes (% of pollen with collapsed walls)	Mean	6.4	5.0	0 - 70
	Range	0 - 30	0 - 55	
	Confidence Interval	1.9 - 13.1	1.2 - 11.2	
	Adjusted P-Value	--	0.533	
	P-Value	--	0.353	
Pollen Viability-Shape, 30 minutes (% of pollen with collapsed walls)	Mean	62.7	60.3	0 - 100
	Range	0 - 100	0 - 100	
	Confidence Interval	38.2 - 84.1	35.9 - 82.2	
	Adjusted P-Value	--	0.621	
	P-Value	--	0.456	
Pollen Viability-Shape, 60 minutes (% of pollen with collapsed walls)	Mean	85.4	83.2	5 - 100
	Range	20 - 100	5 - 100	
	Confidence Interval	NA	NA	
	Adjusted P-Value	--	0.621	
	P-Value	--	0.497	
Pollen Viability-Shape, 120 minutes (% of pollen with collapsed walls)	Mean	98.2	97.1	5 - 100
	Range	75 - 100	35 - 100	
	Confidence Interval	NA	NA	
	Adjusted P-Value	--	NA	
	P-Value	--	NA	
Pollen Viability-Color, 0 minutes (% of pollen yellow in color)	Mean	8.2	6.9	0 - 60
	Range	0 - 60	0 - 55	
	Confidence Interval	3.3 - 14.9	2.5 - 13.2	
	Adjusted P-Value	--	0.385	
	P-Value	--	0.154	
Pollen Viability-Color, 30 minutes (% of pollen yellow in color)	Mean	64.2	60.7	0 - 100
	Range	5 - 100	0 - 100	
	Confidence Interval	40.9 - 84.4	37.4 - 81.7	
	Adjusted P-Value	--	0.533	
	P-Value	--	0.319	
Pollen Viability-Color, 60 minutes (% of pollen yellow in color)	Mean	87.0	84.3	10 - 100
	Range	40 - 100	5 - 100	
	Confidence Interval	NA	NA	
	Adjusted P-Value	--	0.533	
	P-Value	--	0.356	
Pollen Viability-Color, 120 minutes (% of pollen yellow in color)	Mean	98.6	97.6	25 - 100
	Range	75 - 100	35 - 100	
	Confidence Interval	NA	NA	
	Adjusted P-Value	--	NA	
	P-Value	--	NA	

Table 34. Across-Site Analysis of Agronomic Characteristics Results (continued)

Agronomic Characteristic	Reported Statistics	Control Maize	DP23211 Maize	Reference Data Range
Plant Height (cm)	Mean	246.4	249.3	124.8 - 295.0
	Range	155.2 - 300.8	161.0 - 292.0	
	Confidence Interval	223.5 - 269.3	226.4 - 272.2	
	Adjusted P-Value	--	0.342	
	P-Value	--	0.114	
Days to Maturity	Mean	119.7	120.1	105 - 132
	Range	105 - 134	105 - 134	
	Confidence Interval	114.7 - 124.7	115.1 - 125.1	
	Adjusted P-Value	--	0.457	
	P-Value	--	0.213	
Lodging	Mean	4.2	4.3	0.0 - 51.0
	Range	0.0 - 25.9	0.0 - 26.9	
	Confidence Interval	1.4 - 8.4	1.4 - 8.5	
	Adjusted P-Value	--	0.987	
	P-Value	--	0.931	
Final Population (count/m^2)	Mean	5.9	5.7	4.1 - 6.6
	Range	4.3 - 6.6	3.7 - 6.4	
	Confidence Interval	5.6 - 6.2	5.4 - 6.0	
	Adjusted P-Value	--	0.00140†	
	P-Value	--	0.000280*	
Dropped Ears (count)	Mean	0.1	0.1	0 - 7
	Range	0 - 1	0 - 1	
	Confidence Interval	NA	NA	
	Adjusted P-Value	--	NA	
	P-Value	--	NA	
Harvest Grain Moisture (%)	Mean	18.8	18.9	12.2 - 26.1
	Range	13.1 - 25.7	13.4 - 27.3	
	Confidence Interval	16.7 - 21.0	16.7 - 21.0	
	Adjusted P-Value	--	0.987	
	P-Value	--	0.987	
Yield (bushels/A)	Mean	181.5	172.6	16 - 275
	Range	28 - 260	29 - 255	
	Confidence Interval	145.7 - 217.3	136.8 - 208.4	
	Adjusted P-Value	--	0.132	
	P-Value	--	0.0351*	
100-Kernel Weight (g)	Mean	33.2	33.3	19.3 - 44.1
	Range	19.3 - 42.5	19.8 - 39.7	
	Confidence Interval	30.3 - 36.1	30.4 - 36.2	
	Adjusted P-Value	--	0.928	
	P-Value	--	0.805	

Note: NA (not applicable): mixed model analysis was not performed.
* A statistically significant difference (P-Value <0.05) was observed.
† Adjusted P-Value <0.05 was observed.

VIII-B.2. Efficacy Evaluation of DP23211 Maize

Expression of DvSSJ1 dsRNA and IPD072Aa protein in DP23211 maize provides control of certain coleopteran pests such as CRW. Field efficacy testing was conducted during the 2018 growing season at 14 sites in commercial maize-growing regions of North America (two sites in Illinois, two sites in Indiana, four sites in Iowa, two sites in Minnesota, two sites in Nebraska, one site in South Dakota, and one site in Wisconsin). Each field location utilized single-row plots that were 10 feet (3.0 m) in length, arranged in a randomized complete block design with three replications. Non-genetically modified, non-GE near-isoline maize was included as a negative control.

When plants reached V2-V4 growth stage, plots were manually infested with non-diapausing WCR eggs at a rate of 750-1500 eggs per plant, depending on location (Table 35). Injury from larval feeding on roots was evaluated between 56 and 78 days after planting, when plants were at the R2 growth stage. Root injury was recorded using the Iowa State 0-3 node-injury scale (Table 36). No efficacy data were collected at five of the locations due to a low nodal injury score on non-GE near-isoline negative control maize roots. Despite manual infestation, low nodal injury scores (< 0.75 node pressure) at these sites were due to low populations of CRW. Statistical analysis was conducted using a linear mixed model to evaluate and compare node-injury results for DP23211 maize and the control maize.

Mean node-injury score for DP23211 maize was significantly lower than that of the non-GE near isoline negative control maize (p-value <0.0001), demonstrating that DP23211 maize was efficacious against CRW (Table 37).

Methods for efficacy evaluation are presented in Appendix 12. Materials and Methods for Efficacy Evaluation.

Table 35. Field Efficacy Testing Locations and Infestation Rates

Location	Infestation Rate
Mansfield, Illinois, USA	750 eggs/plant
Seymour, Illinois, USA [a]	1500 eggs/plant
Fowler, Indiana, USA [a]	750 eggs/plant
Goodland, Indiana, USA [a]	750 eggs/plant
Johnston #1, Iowa, USA	750 eggs/plant
Johnston #2, Iowa, USA	1500 eggs/plant
Marion, Iowa, USA	750 eggs/plant
Readlyn, Iowa, USA	1500 eggs/plant
Benson, Minnesota, USA	750 eggs/plant
Mankato, Minnesota, USA	750 eggs/plant
York, Nebraska, USA [a]	1500 eggs/plant
Lindsay, Nebraska, USA	750 eggs/plant
Brookings, South Dakota, USA	750 eggs/plant
Janesville, Wisconsin, USA [a]	1500 eggs/plant

[a] Data were not collected at five sites (Seymour, Fowler, Goodland, York, and Janesville) due to a low nodal injury score for the non-GE near isoline negative control maize.

Table 36. Iowa State 0-3 Node-Injury Root Rating Scale

0.00	No feeding damage (lowest rating that can be given)
1.00	One node (circle of roots), or the equivalent of an entire node, eaten back to within approximately 1 ½ inches of the stalk (soil line of the 7th node)
2.00	Two nodes completely pruned
3.00	Three or more nodes completely pruned (highest rating that can be given)
	Damage between complete nodes eaten is noted as the percentage of the total nodes of roots that has been eaten: How much of one node is eaten? Record: ¾ (0.75) ½ (0.50) ¼ (0.25) (0.10) One root pruned anywhere on root system (0.09) Severe feeding scars (no pruning) (0.02) Light-moderate feeding scare (no pruning) (0.00) No feeding evident (perfect root)

Table 37. DP23211 Maize Efficacy Results Against Corn Rootworm

Maize Line	Number of Plots	Mean Node-Injury Root Rating ± SD	Range	P-Value
DP23211	27	0.13 ± 0.08	0.02 – 0.70	<0.0001[a]
Control	27	1.79 ± 0.74	0.50 – 3.00	

[a] Statistically significant difference (P-value < 0.05)

VIII-B.3. Biotic and Abiotic Stressor Measurement

DP23211 maize has been evaluated for response to biotic and abiotic stressors in field tests located in the United States, United States territories, and Canada.

Experiment A – 2018 Field Trial Biotic and Abiotic Stressor Measurement

Data were collected from 12 sites in conventional maize-growing regions of the United States (one site in Indiana, Minnesota, Nebraska, and Pennsylvania; two sites in Illinois and Texas; and three sites in Iowa) and Canada (one site in Ontario) during the 2018 growing season. Each block in this experiment contained DP23211 maize, non-GE isoline control maize, and four reference maize lines planted in 6-row plots at a rate of 30 seeds per row. Each row was 20 ft (6.1 m) in length and 30 in. (76 cm) in width (with the exception of site RG086TX1 where row width was 39 in.). Each block was separated by an alley of at least 3 ft. (0.9 m) in width, and each plot was bordered on either side by one row of maize.

For each trial site, a survey of the naturally occurring insects, diseases, and abiotic stressors were recorded at four observation periods. These observations provide a means to determine if DP23211 maize will respond differently from conventional maize lines to insects (other than CRW), diseases, abiotic stressors in the environment.

Observations from field trials demonstrated that DP23211 maize did not exhibit any unexpected responses to naturally occurring insects (other than CRW) or diseases, and abiotic stressors as summarized in Appendix 13. Materials and Methods for Field Insect and Disease Observations. These results support the conclusion that DP23211 maize is comparable to control maize lines with similar genetics or to conventional maize lines with respect to insect (other than CRW), disease, and abiotic stressor response.

Experiment B – 2009-2017 Field Observation Data

DP23211 maize has been field tested in the United States and Puerto Rico over 4 years, as authorized by USDA-APHIS permits and notifications. For each trial, a survey of the naturally occurring insects and diseases and any unexpected differences in the response of DP23211 maize as compared to the control line (near-isoline and/or conventional maize lines) were recorded by experienced plant breeders and field staff at least every four weeks. A summary of these surveys for each trial and any differences seen between DP23211 maize and control lines are presented in Appendix 13. Materials and Methods for Field Insect and Disease Observations. These observations provide a means to determine if DP23211 maize will respond differently from conventional maize lines to insects or diseases in the environment.

In every case, DP23211 maize did not exhibit any unexpected responses to naturally occurring insects (other than CRW) or diseases. These results, taken with the results presented above, support the conclusion that DP23211 maize is comparable to control maize lines with similar

genetics or to conventional maize lines with respect to insect (other than CRW) or disease response.

VIII-C. Conclusions on Agronomic Performance and Field Observations of DP23211 Maize

DP23211 maize was observed in laboratory experiments and at 12 field locations in the United States and Canada to measure agronomic parameters and abiotic and biotic stressors. These experiments and field studies evaluate the characteristics of maize over a broad range of environmental conditions that represent regions where DP23211 maize will be grown. The agronomic parameters measured are characteristic traits for reproduction, survival, and potential weediness.

Agronomic data demonstrated no significant differences between DP23211 maize and control maize (non-GE near-isoline controls and/or conventional maize lines) with respect to early population, vegetative growth, reproductive parameters, yield, and pest responses (Table 34). Statistically significant differences were identified in days to flowering and final population, with 39 of 47 observations for days to flowering and 46 of 47 observations for final population within the respective reference ranges for each agronomic characteristic, indicating that these differences are not likely biologically relevant (Table 34). These data support the conclusion that DP23211 maize is agronomically comparable to conventional maize (Anderson *et al.*, 2020).

Observations from United States and United States territory field trials over multiple years showed no unexpected differences in the response of DP23211 maize and control maize to naturally occurring insects and diseases (VIII-B.3. Biotic and Abiotic Stressor Measurement). These results support the conclusion that DP23211 maize is comparable to control maize lines with similar genetics and/or to conventional maize lines.

Based on these analyses, DP23211 maize is comparable to conventional maize and is unlikely to pose a greater plant pest risk or increased weed potential compared to non-GE conventional maize.

IX. Environmental Risk Assessment of DP23211 Maize

As previously stated, DP23211 maize expresses the DvSSJ1 dsRNA and the IPD072Aa protein for control of corn rootworm. DP23211 maize also expresses the PAT protein for tolerance to glufosinate herbicide, and the PMI protein, which was used as a selectable marker. The PAT and PMI proteins present in DP23211 maize are identical to the corresponding proteins found in previously registered events that are currently in commercial use. The mode of action and safety of the PAT and PMI proteins have already been assessed (CERA, 2011b; Hérouet et al., 2005; OECD, 1999; Reed et al., 2001).

An environmental risk assessment (ERA) for the cultivation of DP23211 maize in the United States was conducted based on the risk assessment framework described by the U.S. Environmental Protection Agency (US-EPA, 1998). This framework is currently used to assess GE crops derived from *Bacillus thuringiensis* (*Bt*), and it is robust and suitable for assessing plants expressing non-*Bt* proteins (Anderson et al., 2018) and plants expressing insecticidal RNA (CERA, 2011a). The DP23211 maize ERA used problem formulation to guide the exposure assessment, hazard assessment, and risk characterization. The ERA is focused on the insecticidal DvSSJ1 dsRNA and the IPD072Aa protein expressed by DP23211 maize and assessed the potential exposure and hazard to non-target organisms (NTOs). Potential pathways to harm related to weediness and outcrossing were also considered.

IX-A. Problem Formulation

Problem formulation was used to develop hypotheses of potential harm to NTOs and the environment, based on knowledge of the receiving environment, the biology of the crop, and the characteristics of the introduced insecticidal traits. Problem formulation was used to guide the exposure and hazard assessments, so that the ERA is informative and predictive of risk (Carstens et al., 2010; Raybould, 2006; Romeis et al., 2013; Wolt and Peterson, 2010).

IX-A.1. Biology of the Crop and Receiving Environment

Several characteristics related to the biology of the crop and the receiving environment were considered as part of problem formulation to develop potential pathways to harm related to weediness or outcrossing. The biology of unmodified cultivated maize (*Zea mays* L.), has been described in the documents published by the Organisation for Economic Co-operation and Development (OECD, 2003). These documents contain the information pertaining to aspects of maize biology, including: taxonomy and morphology, use as a crop plant, agronomic practices, center of origin, reproductive biology, potential to be a volunteer weed, possibility and consequences of interspecific and intergeneric crosses with wild relatives, interaction with other organisms, and a summary of ecology.

IX-A.1.a. Potential for Weediness

Maize is extensively cultivated worldwide and has a long history of safe use. Conventional maize is well-established as having low weediness and invasiveness potential, as it is highly domesticated, is unlikely to establish itself in self-sustaining populations outside of cultivation, and is a poor competitor with native vegetation. Maize seeds show poor dormancy (CFIA, 1994) and generally only survive under favorable climatic conditions. Maize is an annual plant that lacks seed dormancy, which limits survival from one growing season to the next (Andersson and de Vicente, 2010; CFIA, 1994). Therefore, the natural characteristics of maize do not indicate a high potential for weediness or invasiveness.

IX-A.1.b Potential for Gene Flow

The potential for gene flow between a GM crop and its sexually compatible wild relatives is assessed through several factors. One factor includes the potential for pollen flow and outcrossing to occur outside the cultivated field. Other factors include the overlap of the wild relative's geographic distribution with the region of GM crop cultivation and the possibility of genetic compatibility between the crop and the relative. Finally, to determine the potential for widespread introgression of the trait into wild relative populations, the potential for the trait to alter weediness characteristics and the weediness of the wild relative are considered.

Maize has a high outcrossing rate and can pollinate sexually compatible varieties (e.g., other cultivated maize hybrids, teosinte) (OECD, 2003). However, gene flow in the environment is limited by environmental barriers (pollen viability, pollen dispersal, proximity and synchrony of flowering) (Andersson and de Vicente, 2010; CFIA, 1994; Luna et al., 2001; Messeguer et al., 2006) and genetic barriers (ability to outcross and produce fertile progeny) (OECD, 2003).

Maize is almost entirely cross-fertilizing and its pollen is typically wind dispersed (OECD, 2003). Millions of pollen grains are produced per plant (Jarosz et al., 2005). Despite pollination characteristics that are favorable for pollen flow, other factors make it highly unlikely that viable maize pollen will travel significantly outside of the cultivated field. Pollen viability is reduced in a matter of hours under high temperature and low humidity (Aylor, 2004). Studies also indicate that the majority of maize pollen is unlikely to be dispersed significant distances outside the originating field (Jarosz et al., 2003). Numerous studies show the majority (84-92%) of pollen grains travel less than five meters (Pleasants et al., 2001), with nearly all (>99.75%) pollen traveling less than 100 meters (Byrne and Fromherz, 2003; Matsuo et al., 2004; Sears and Stanley-Horn, 2000). The potential of cross-pollination between cultivated maize and its wild relatives will be highest where the wild relatives grow near or adjacent to areas of cultivation. Therefore, the geographic range of wild relatives compared to the regions of maize cultivation is one critical factor in determining the potential for gene flow.

Taxonomically, maize (*Zea mays* L.) is a member of the *Maydeae* tribe of the grass family, *Poaceae* (OECD, 2003). Teosinte, within the genus *Zea*, and the genus *Tripsacum* are the closest relatives to maize taxonomically. The genus *Tripsacum* is also included in the *Maydeae* tribe (OECD, 2003). Annual teosintes are grouped into the species *Zea mays*, although there is some dispute of this classification based on characteristics that prevent a high degree of introgression (OECD, 2003). Annual teosintes have been further classified into the subspecies *Zea mays* ssp. *mexicana* and *Zea mays* ssp. *parviglumis* (OECD, 2003). In contrast, perennial teosintes are classified as different species altogether: *Zea perennis* and *Zea diploperennis* (OECD, 2003). Both annual and perennial teosintes are considered the closest wild relatives of cultivated maize (OECD, 2003). Perennial plants of the genus *Tripsacum* are considered the next closest relatives of maize (OECD, 2003). Neither the *Zea* genus nor the *Tripsacum* genus are listed as noxious weeds on the federal or state noxious weed lists (USDA-NRCS, 2011).

Both annual and perennial teosintes are normally confined to the tropical and subtropical regions of Mexico, Honduras, Guatemala, and Nicaragua (Iltis, 2011). In the U.S., sparsely dispersed introduced populations of annual teosintes *Zea mexicana* (synonym: *Zea mays* ssp. *mexicana*) and *Zea mays* ssp. *parviglumis* have been reported in Florida, Maryland, and Alabama (USDA, 2011). Also, an isolated population of *Zea perennis* (perennial teosinte) has been introduced in South Carolina (USDA, 2011). While maize can hybridize with these species under natural conditions, there is incompatibility between some maize populations and certain types of teosinte that results in low fitness of some hybrids and prevents a high rate of introgression (OECD, 2003). Together with the very limited geographic range of the teosinte population in the U.S., the probability of gene flow from cultivated maize fields to these wild relatives is very low.

Plants of the genus *Tripsacum* are mostly found in Mexico, Central, and South America (OECD, 2003). Three of these species (*T. dactyloides, T. floridanum*, and *T. lanceolatum*) exist as native species populations in the continental U.S., and two species (*T. fasciculatum* and *T. latifolium*) were introduced in Puerto Rico (USDA, 2011). *T. dactyloides* occurs throughout the eastern half of the U.S. *T. lanceolatum* occurs in Arizona and New Mexico (USDA, 2011) and *T. floridanum* is native to southern Florida (USDA, 2011). Although it is extremely difficult, *Tripsacum* species (*T. dactyloides*, *T. floridanum*, and *T. lanceolatum*) can be crossed with maize; however, hybrids have a high degree of sterility and are genetically unstable (OECD, 2003). Successful crosses of maize with *Tripsacum* species have been made experimentally, however such crosses are not known to occur in the wild (OECD, 2003). Therefore, gene flow between cultivated maize and relatives of the genus *Tripsacum* is highly unlikely.

IX-A.2. Characteristics of the Introduced Insecticidal Traits

Several characteristics related to the introduced insecticidal traits were considered as part of problem formulation (e.g., insecticidal modes of action, potential for interaction between the insecticidal traits, similarity with previously assessed traits, specificity of the traits). These characteristics are discussed further below for DvSSJ1 dsRNA and the IPD072Aa protein expressed by DP23211 maize.

IX-A.3. Potential for Interaction between DvSSJ1 dsRNA and the IPD072Aa Protein

The potential for interaction, as it relates to synergism, between the DvSSJ1 dsRNA and the IPD072Aa protein was evaluated using the framework outlined by EPA (US-EPA, 2009). As described further below (section XII. Analysis of Potential Synergism of DvSSJ1 dsRNA and IPD072Aa Protein in DP23211 Maize), the DvSSJ1 dsRNA and the IPD072Aa protein have different modes of action. Therefore, the independent model of combined action (Bliss, 1939) was used to estimate the potency of a mixture of DvSSJ1 dsRNA and the IPD072Aa protein. The results showed no evidence of biologically relevant synergistic effects between the DvSSJ1 dsRNA and the IPD072Aa protein, based on the EPA framework (US-EPA, 2009). Thus, safety studies conducted individually on the DvSSJ1 dsRNA or the IPD072Aa protein can be used to inform the ERA of DP23211 maize.

IX-A.4. Similarity to Previously Assessed Traits

RNA interference (RNAi) is a naturally occurring mechanism for down-regulation of gene expression in most plants and animals, and it involves a process that promotes an RNA transcripts degradation when dsRNA is endogenously transcribed or exogenously introduced into a cell with a sequence that is complementary to the mRNA produced by transcription of a gene. There are many reviews that describe the RNAi machinery and provide a detailed mechanism of action of RNAi (for example, Fire et al., 1998; Kurreck, 2009; Mello and Conte, 2004; Price and Gatehouse, 2008). Several GE crops have, or are being developed using RNAi to improve taste or nutritional profile, provide resistance to viruses, and to control of insect pests (for example, Anderson et al., 2016; Baum et al., 2007; Bonfim et al., 2007; Krieger et al., 2008; Mao et al., 2011; Pavely et al., 2007). Therefore, the use of RNAi in the field of agricultural biotechnology has a history of safety, and the established ERA framework for GE crops is robust and suitable for assessing plants developed using RNAi (CERA, 2011a). The DvSSJ1 trait produces dsRNA, which down-regulates expression of the DvSSJ1 protein in the mid-gut of the WCR. It will be shown in Section IX-B.2.b. Feeding Bioassay Assessment of DvSSJ1 dsRNA Specificity below that DvSSJ1 dsRNA is specific, with activity limited to species within the genus *Diabrotica* and the family Chrysomelidae.

The IPD072Aa protein is derived from *Pseudomonas chlororaphis* (Schellenberger et al., 2016). *P. chlororaphis* is a naturally occurring, ubiquitous bacterium found in the environment that lacks known allergenic or toxic properties and has a history of safe use in agriculture (Anderson et al.,

2018). Certain *Pseudomonas* species, including *P. chlororaphis,* have been used in agriculture as seed treatments, foliar-applied biopesticides for fungal and disease control, and as a gene source for GM crops. Over the past 30 years, the US EPA has registered several *Pseudomonas*-based biopesticides and granted exemptions from the requirements of a tolerance (e.g. 40 CFR 180-1114, 180.1145, 1802.1212) further demonstrating the history of safe use with this source organism (Anderson et al., 2018). The established ERA framework that is currently used to assess GM crops derived from *Bt* is robust and suitable for assessing plants expressing non-*Bt* proteins (Anderson et al., 2018). It will be shown in section IX-C.2.a. Feeding Bioassay Assessment of IPD072Aa Protein Specificity that the IPD072Aa protein is specific, with activity limited to within the order Coleoptera.

IX-B. Analysis of DvSSJ1 dsRNA Specificity

When DP23211 plants expressing DvSSJ1 dsRNA are ingested by WCR, production of the DvSSJ1 protein in the intestinal lining is suppressed. Reduction in the DvSSJ1 protein, and the subsequent loss of the gut epithelial barrier and cellular deformities, is lethal to WCR (Hu et al., 2016; Hu et al., 2019). Since the mode of action is sequence based, bioinformatics analyses were combined with spectrum of activity insect bioassays for DvSSJ1 dsRNA to support the conclusion that the activity of DvSSJ1 dsRNA is limited to the genus *Diabrotica*.

The results of the bioinformatics analysis show that the sequence of the DvSSJ1 dsRNA is identical to the WCR *dvssj1* gene (100% sequence match), and high-percentage matches to additional *Diabrotica* species (presented in section IX-B.2.a. Bioinformatic Assessment of DvSSJ1 dsRNA Specificity below). Based on these results, DvSSJ1 dsRNA activity is expected on the target pest, WCR. The high-percentage matches to additional *Diabrotica* species were further investigated with insect diet bioassays as presented in Section IX-B.2.b. Feeding Bioassay Assessment of DvSSJ1 dsRNA Specificity.

An additional *in silico* approach was used to evaluate the species-specificity of DvSSJ1 dsRNA and investigate the potential for off-target effects in humans, livestock, and companion animals. These analyses did not yield any exact 21-nt matches between the sense or anti-sense DvSSJ1 dsRNA fragment sequences and any human or animal transcript, and did not indicate a potential for off-target effects (section IX-B.2.a.1. Bioinformatic Assessment of Humans, Livestock, and Companion Animals).

Spectrum of activity laboratory insect bioassays were conducted with 11 species from four families within the order Coleoptera to determine the spectrum of activity of DvSSJ1 dsRNA. The objective of each bioassay was to evaluate the biological response of each test organism when fed an artificial diet containing DvSSJ1 210bp dsRNA.

The results of these bioassays are presented in section IX-B.2.b. Feeding Bioassay Assessment of DvSSJ1 dsRNA Specificity. Results show that WCR was the most sensitive species tested, with an LC_{50} of 0.036 ng DvSSJ1 dsRNA/mg(Sturtz *et al.*, 2018)(Sturtz *et al.*, 2018). The only other species showing sensitivity to DvSSJ1 dsRNA was southern corn rootworm (SCR), which was observed to have decreased survival at concentration 100 ng DvSSJ1 dsRNA/ml diet.

Therefore, based on the specificity of the DvSSJ1 dsRNA and the IPD072Aa protein, the ERA for DP23211 maize is primarily focused on non-target coleopterans.

Further detail regarding the DvSSJ1 dsRNA mode of action and molecular target, bioinformatics assessment, and inect bioassays are presented in Sections IX-B.1. and IX-B.2. below.

IX-B.1. DvSSJ1 dsRNA Mode of Action and Molecular Target

DP23211 maize plants expressing DvSSJ1 dsRNA targeting WCR *DvSSJ1* mRNA*,* show insecticidal activity and plant protection from WCR damage. The *dvssj1* gene encodes a membrane protein associated with the smooth sepate junction (SSJ) which is required for intestinal barrier function. Smooth septate junctions (SSJs) are occluding junctions comprising a network of proteins that physically connect adjacent cells. These junctions are found in insect midgut epithelial cells in arthropods, and have identified roles in intestinal barrier function and paracellular transport in the renal system (Furuse and Izumi, 2017; Hu et al., 2016). The *dvssj1* gene identified in WCR is an ortholog of the snakeskin (*ssk*) gene, originally identified in the *Drosophila* midgut, and its protein product is a critical component of the SSJ protein complex (Hu et al., 2016; Yanagihashi et al., 2012). Relative *dvssj1* mRNA expression is highest in WCR neonates, and *dvssj1* mRNA and protein localize to the midgut. When DP23211 maize is ingested by the WCR, plant-derived DvSSJ1 dsRNA is processed into 21 nucleotide (21-nt) small interfering RNAs (siRNAs) which downregulates *dvssj1* mRNA and decreases translation of DvSSJ1 protein. Loss of DvSSJ1 protein in the WCR midgut disrupts the SSJ protein complex, leading to loss of barrier integrity, growth inhibition and larval mortality (Hu et al., 2019).

The expression of the *DvSSJ1* silencing cassette results in the full-length transcript of 901 nucleotides containing a 210 bp inverted fragment of the *DvSSJ1* gene, the formation of a dsRNA transcript and siRNAs in transgenic plants. Detection of DvSSJ1 siRNAs via small RNA sequencing in DP23211 maize plants showed that the predominant species of siRNA to be the 21-nt fragment, consistent with previous findings in GE plants containing RNAi constructs (Hu, et al., 2020; Baum, et al., 2007; Li, et al., 2015). The siRNAs detected in DP23211 maize generally aligned across the 210bp-length of the DvSSJ1 fragment (Hu et al., 2020).

The specificity of the DvSSJ1 dsRNA and its relation to insecticidal activity has also been investigated. A 21 bp DvSSj1 siRNA, was selected (based on previous feeding study results (Hu, et al., 2019)), to determine what level of complementarity was necessary to see insecticidal

effects in WCR. Single nucleotide polymorphisms (SNPs) were introduced into varying locations in the 21-mer DvSSJ1 siRNA and fed to WCR in a 14-day bioassay. When compared against an unaltered DvSSJ1 21-mer, a 1 bp point mutation reduced WCR mortality by >10% (68% unaltered; 55% 1 bp mutation). The introduction of a 2 bp mutation in to the 21-mer at 2 locations, reduced WCR mortality to 20%, and the downward trend in WCR mortality continued as more SNPs at additional locations were added. Results showed that the number and location of the SNPs are both important factors in determining the insecticidal activity of DvSSJ1 dsRNA (Hu, et al., 2020).

Additionally, comparison of homologous sequences of DvSSJ1 from other insect groups is presented in Section IX-B.2.a. Bioinformatic Assessment of DvSSJ1 dsRNA Specificity and shows percent similarity, number of SNPs, and the number of 21 nt matches (Table 38). The information presented for other insect species, in combination with DvSSJ1 dsRNA bioassay feeding study results (presented in Section IX-B.2.b. Feeding Bioassay Assessment of DvSSJ1 dsRNA Specificity), demonstrates that the molecular target of 210 bp DvSSJ1 dsRNA is specific to the *Diabrotica* genus in the Chrysomelidae family of Coleoptera.

The form of the insecticidally active molecule of DvSSJ1 RNA was determined by comparing GE plants expressing 21 bp siRNA of DvSSJ1 against GE plants expressing 210bp DvSSJ1 dsRNA and dicer processed siRNAs. Plants were infested with WCR eggs and were scored for feeding damage 3 weeks later. The nodal injury scores showed a significant reduction of damage for plants expressing the 210bp DvSSJ1 dsRNA as compared to GE plants expressing the 21bp siRNA and dicer processed siRNA (Hu, et al., 2020).

These results support the conclusion that the 210bp DvSSJ1 dsRNA is the functional molecule for insecticidal action in DP23211 maize and this molecule should be used for insect feeding bioassays to assess specificity and effects to NTOs. Additionally, a single nucleotide mutation of a 21-mer that maps to the 210 bp DVSSJ1 dsRNA does reduce the effectiveness on WCR mortality, indicating that exact matching is necessary for activity against WCR.

IX-B.2. DvSSJ1 dsRNA Spectrum of Activity and Species Specificity

Early in the characterization process, an *in silico* comparative bioinformatics approach was used to assess how conserved the *ssj1* gene sequence is across different organisms with varied evolutionary distance from WCR. The sequences of the *ssj1* homologs from twenty species, representing four families within the order Coleoptera, four families within the order Lepidoptera, and two additional non-target organisms, were compared to a 210-base pair (210-bp) sequence from the *dvssj1* gene (referred to as 210-bp *dvssj1* sequence) to determine the percent similarity, number of single nucleotide polymorphisms (SNPs), and the number of 21-nt matches (Volume 2, Appendix A). An additional *in silico* approach was used to evaluate the

species-specificity of DvSSJ1 dsRNA and investigate the potential for off-target effects in humans, livestock, and companion animals (Volume 2, Appendix B and C).

As shown in Section IX-B.2.a. Bioinformatic Assessment of DvSSJ1 dsRNA Specificity, the sequence of the DvSSJ1 dsRNA is specific to WCR, and may have activity on other *Diabrotica* species (SCR and NCR). Therefore, the spectrum of activity of the DvSSJ1 dsRNA was assessed in insect feeding bioassays and those assays focused on species from Coleoptera and Lepidoptera where activity could be expected. Lepidoptera were also assessed to determine potential for cross-order activity. Ten species from four families within the order Coleoptera (Chrysomelidae, Tenebrionidae, Coccinellidae, and Staphylinidae), and four species from four families within the order Lepidoptera (Crambidae, Tortricidae, Nymphalidae, and Noctuidae) were used in DvSSJ1 dsRNA feeding bioassays (Boeckman, 2019, in preparation).

An additional *in silico* approach was used to evaluate the species-specificity of DvSSJ1 dsRNA and investigate the potential for off-target effects in humans, livestock, and companion animals. Results are presented in Section IX-B.2.a.1. Bioinformatic Assessment of Humans, Livestock, and Companion Animals below.

IX-B.2.a. Bioinformatic Assessment of DvSSJ1 dsRNA Specificity

A bioinformatics approach was used to assess how conserved the *ssj1* gene sequence is across different organisms with varied evolutionary distance from WCR. The sequences of the *ssj1* homologs of twenty species, representing four families within the order Coleoptera, four families within the order Lepidoptera, and two additional non-target organisms were compared to a 210-base pair (210-bp) sequence from the *dvssj1* gene (referred to as 210-bp *dvssj1* sequence) to determine the percent similarity, number of single nucleotide polymorphisms (SNPs), and the number of 21 nucleotide (21-nt) matches. *In silico* analysis was primarily focused within the order Coleoptera, with a total of 13 species assessed, because the 210-bp DvSSJ1 dsRNA is designed to match a segment of the messenger RNA (mRNA) sequence from the WCR smooth septate junction protein 1 (*dvssj1*) gene (Volume 2, Appendix D, Section D1.)

Within the family Chrysomelidae, species tested include the WCR, northern corn rootworm (NCR; *Diabrotica barberi*), southern corn rootworm (SCR; *Diabrotica undecimpunctata*), crucifer flea beetle (CFB, *Phyllotreta cruciferae*), striped flea beetle (SFB, *Phyllotreta striolata*), and Colorado potato beetle (CPB; *Leptinotarsa decemlineata*). Within the family Tenebrionidae, species tested included: red flour beetle (RFB, *Tribolium castaneum*), superworm (SWM; *Zophobas morio*), and mealworm (MWM; *Tenebrio molior*). Within the family Staphylinidae, the species tested was rove beetle (RVB; *Dalotia coriaria*). Within the family Coccinellidae, species tested included: the Mexican bean beetle (MBB; *Epilachna varivestis*), mealy bug destroyer (MBD; *Cryptolaemus montrouzieri*), and the pink spotted lady beetle (CMAC; *Coleomegilla maculata*). The sequences of seven species outside the order Coleoptera were also assessed to test for potential cross-order

activity based on nucleotide match to the 210-bp *dvssj1* sequence. Five species within the order Lepidoptera, including: painted lady butterfly (PL; *Vanessa cardui*), European corn borer (ECB, *Ostrinia nubilalis),* fall armyworm (FAW, *Spodoptera frugiperda*), codling moth (CDM; *Cydia pomonella),* and corn earworm (CEW, *Helicoverpa zea*), as well as two non-target organisms (honeybee (*Apis mellifera*) and the insidious flower bug (*Orius insidiosus)*) were also assessed.

Using bioinformatics analysis, the closest sequence match (percent identity to the 210-bp *dvssj1* sequence) was the *ssj1* homologous gene from WCR, which as intended had a 100% sequence match, 0 SNPs, and 190 21-nt matches (Table 38). The *ssj1* homologous gene from the closely related species, NCR, shared 97.1% identity with the 210-bp *dvssj1* sequence, with 6 SNPs and 135 21-nt matches. The *ssj1* homologous gene from SCR shared 92.9% identity with the 210-bp *dvssj1* sequence, with 15 SNPs and 79 21-nt matches. The *ssj1* homologous genes from the other Coleoptera within the family Chrysomelidae (CFB, SFB, and CPB) as well as species within the family Tenebrionidae, the family Coccinellidae, and the family Staphylinidae had decreasing percent identity with the 210-bp *dvssj1* sequence, ranging from 77.6 to 61.9% similarity and an increasing number of SNPs (ranging from 47 to 80). All Lepidoptera species within the four families, as well as the honey bee (*Apis mellifera)* and the insidious flower bug (*Orius insidiosus)* also had lower percent identity with the 210-bp *dvssj1* sequence, ranging from 68.1 to 60% similarity, and an increased number of SNPs (ranging from 67 to 84). There were zero of 21-nt matches observed across all of the non-*Diabrotica* species analyzed.

Methods used for the bioinformatic analysis of DvSSJ1 (inclusive of sequences used in the analysis) are presented in Volume 2, Appendix A.

Table 38. Sequence Comparison of *ssj1* Homologs

Order	Family	Species	Common name	Percent (%) Identity to 210-bp *dvssj1*	Number of SNPs	# of 21-nt matches (longest sequence)
Coleoptera	Chrysomelidae	*Diabrotica virgifera virgifera*	Western corn rootworm	100	0	190
		Diabrotica barberi	Northern corn rootworm	97.1	6	135
		Diabrotica undecimpunctata	Southern corn rootworm	92.9	15	79
		Phyllotreta Cruciferae	Crucifer flea beetle	77.6	47	0 (20)
		Phyllotreta striolata	Striped flea beetle	76.2	50	0 (19)
		Leptinotarsa decemlineata	Colorado potato beetle	73.3	56	0 (12)
	Tenebrionidae	*Tribolium castaneum*	Red flour beetle	69.5	64	0 (11)
		Zophobas morio	Super worm	69	65	0 (10)
		Tenebrio molior	Mealworm	65.2	73	0 (10)
	Staphylinidae	*Dalotia coriaria*	Rove beetle	64.3	75	0 (8)
	Coccinellidae	*Epilachna varivestis*	Mexican bean beetle	67.6	68	0 (12)
		Cryptolaemus montrouzieri	Mealy bug destroyer	63.3	77	0 (8)
		Coleomegilla maculata	Pink spotted lady beetle	61.9	80	0 (13)
Lepidoptera	Nymphalidae	*Vanessa cardui*	Painted lady	64.8	74	0 (8)
	Crambidae	*Ostrinia nubilalis*	European corn borer	64.2	79	0 (9)
	Noctuidae	*Spodoptera frugiperda*	Fall armyworm	62.9	78	0 (11)
	Tortricidae	*Cydia pomonella*	Codling moth	60.5	83	0 (8)
	Noctuidae	*Helicoverpa zea*	Corn earworm	60	84	0 (8)
Hymentoptera	Apidae	*Apis mellifera*	Honeybee	68.1	67	0 (10)
Hemiptera	Anthocoridae	*Orius insidiosus*	Insidious flower bug	61.9	80	0 (11)

IX-B.2.a.1. Bioinformatic Assessment of Humans, Livestock, and Companion Animals

An additional *in silico* approach was used to evaluate the species-specificity of DvSSJ1 dsRNA and investigate the potential for off-target effects in humans, livestock, and companion animals. The DvSSJ1 dsRNA sense and anti-sense fragment sequences were parsed into all possible sequentially-overlapping 21-nt siRNA subsequences, and each resultant sequence was compared to similarly-parsed sequentially-overlapping 21-nt sequences from the transcriptomes of humans, chickens, pigs, cattle, sheep, goats, turkeys, salmon, dogs and cats. These analyses did not yield any exact 21-nt matches between the sense or anti-sense DvSSJ1 dsRNA fragment sequences and any human or animal transcript, and did not indicate a potential for off-target effects. Methods of analysis and bioinformatic output are presented in Volume 2, Appendix B for humans and Volume 2, Appendix C for animals.

IX-B.2.b. Feeding Bioassay Assessment of DvSSJ1 dsRNA Specificity

Laboratory feeding bioassays were conducted with ten species from four families within the order Coleoptera (Chrysomelidae (WCR, SCR, CPB), Tenebrionidae (MWM, SWM, RFB), Staphylinidae (RVB), Coccinellidae (MBB), Convergent Lady Beetle, (CMAC)), and and four species from four families within the order Lepidoptera (Crambidae (ECB), Tortricidae (CDM), Nymphalidae (PL), and Noctuidae (CEW)) to determine the spectrum of activity of DvSSJ1 dsRNA. The objective of each bioassay feeding study was to evaluate the biological response of each test organism when fed an artificial diet containing DvSSJ1 210bp dsRNA.

In DP23211 maize, the 210 bp form of the DvSSJ1 dsRNA is the functional molecule for activity against WCR. As shown in Table 18, expression of DvSSJ1 dsRNA in DP23211 maize is low. Therefore, it was necessary to produce RNA using *in Vitro* techniques in order to obtain enough DvSSJ1 dsRNA to perform safety analyses.

The concentration, purity, and sequence of the *in Vitro* produced DvSSJ1_210_dsRNA test material was assessed to determine if the test material is equivalent for use in DvSSJ1 dsRNA specificity and NTO feeding bioassays. Agarose gel electrophoresis-based densitometric measurements were conducted to determine the DvSSJ1_210 dsRNA concentration by comparison with known concentrations of a DNA standard. Polyacrylamide gel electrophoresis (PAGE)-based densitometric measurements were performed to determine the purity of DvSSJ1_210 dsRNA. The purity of DvSSJ1_210 dsRNA was presented as a percentage of the total densitometric reading from all RNA varieties shown on a gel. To confirm DvSSJ1_210 dsRNA sequence identity, reverse transcription-polymerase chain reaction (RT-PCR) was performed to generate the complementary DNA (cDNA) of DvSSJ1_210 dsRNA. The synthesized cDNA products were cloned into a plasmid for sequencing. Sequencing data from each clone was assembled and compared to the reference sequence.

Results of densitometry analyses confirmed the concentration of the DvSSJ1_210 dsRNA and determined the purity to be 95.1%. The sequence of the full-length DvSSJ1_210 dsRNA was confirmed and shown to be equivalent to the DvSSJ1 dsRNA.

The sequence of the full-length *in Vitro* produced DvSSJ1 210 base pair dsRNA (DvSSJ1_210 dsRNA) was confirmed as equivalent when compared to the DvSSJ1_210 dsRNA reference sequence. This equivalence shows that the *in Vitro* produced DvSSJ1_210 dsRNA is suitable for use in specificity and NTO bioassay feeding assessments.

IX-B.2.b.1. Western Corn Rootworm (WCR)

WCRis known to be sensitive to DvSSJ1 210bp dsRNA (DvSSj1_210). Multiple dose-response bioassays were conducted exposing WCR larvae via oral ingestion to six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 0.0001 ng DvSSJ1_210 ds RNA per mg diet wet weight)
- Treatment 3: Test Diet (targeting 0.001 ng DvSSJ1_210 ds RNA per mg diet wet weight)
- Treatment 4: Test Diet (targeting 0.01 ng DvSSJ1_210 ds RNA per mg diet wet weight)
- Treatment 5: Test Diet (targeting 0.1 ng DvSSJ1_210 ds RNA per mg diet wet weight)
- Treatment 6: Test Diet (targeting 1 ng DvSSJ1_210 ds RNA per mg diet wet weight)

For each bioassay, each diet was provided to 30 individual WCR for a total of 14 days. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 14 days, the bioassays were complete, mortality was assessed, and surviving organisms were individually weighed. The dose-response relationship between the DvSSJ1 210bp dsRNA (DvSSJ1_210 dsRNA) concentration and WCR mortality was statistically analyzed for each bioassay and a median lethal concentration (LC_{50}) was estimated. An overall mean LC_{50} was estimated across the independent bioassays using a meta-analysis approach.

For each bioassay, QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in test dosing solutions used to prepare Treatment 6 and the homogeneity of DvSSJ1_210 dsRNA in Treatment 6, from two days of diet preparation. In addition, the stability under bioassay conditions of Treatment 6 (with and without WCR infestation) was verified. The absence of DvSSJ1_210 dsRNA in Treatment 1, using diets from two days of diet preparation, was also verified.

Bioassays 1, 2, 5, and 6 met the acceptability criteria and results are reported below. Bioassays 3 and 4 did not meet the acceptability criteria and were terminated.

The estimated overall mean LC_{50} for WCR was 0.036 ng DvSSJ1_210 dsRNA per mg diet wet weight with a 95% confidence interval of 0.0066 - 0.065 ng DvSSJ1_210 dsRNA per mg diet (Table 39).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix D, Section D1.

Table 39. Median Lethal Concentration of DvSSJ1_210 dsRNA (ng/mg) for WCR

Bioassay	LC_{50}		
	Point Estimate	Standard Error	95% Confidence Interval
1	0.045	0.019	0.0064 - 0.083
2	0.042	0.027	0 - 0.096
5	0.084	0.033	0.019 - 0.15
6	0.011	0.0077	0 - 0.026
Overall Mean[a]	0.036	0.015	0.0066 - 0.065

Note: Median lethal concentration (LC_{50}) is expressed in ng DvSSJ1_210 dsRNA per mg diet wet weight

[a] The overall mean values as determined with the statistical analysis described in Volume 2, Appendix D, Section D1.

IX-B.2.b.2. Southern Corn Rootworm (SCR)

The objective of this bioassay was to evaluate the survival and weight of SCR when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). SCR was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

SCR larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 0.1 ng DvSSJ1_210 dsRNA per ml diet wet weight)
- Treatment 3: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per ml diet wet weight)
- Treatment 4: Test Diet (targeting 10 ng DvSSJ1_210 dsRNA per ml diet wet weight)
- Treatment 5: Test Diet (targeting 100 ng DvSSJ1_210 dsRNA per ml diet wet weight)
- Treatment 6: Test Diet (targeting 1000 ng DvSSJ1_210 dsRNA per ml diet wet weight)

Each diet was provided to 30 individual SCR for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 7. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between SCR provided Treatment 1 and those provided Treatments 2-6.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in test dosing solutions used to prepare Treatment 5 and the homogeneity and stability under bioassay conditions of DvSSJ1_210 dsRNA in a test diet (Treatment 5). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified.

The results demonstrated the mortality of SCR fed the test diets containing 1 ng and 10 ng DvSSJ1_210 dsRNA per ml diet (Treatments 2 and 3; 13.3% and 10.0%, respectively) was not significantly greater than the mortality of SCR fed the bioassay control diet (Treatment 1; 10.0%) (Table 40).

Statistically significant differences were observed in mortality between SCR fed the test diets containing 100, 1000, and 10000 ng DvSSJ1_210 dsRNA per ml diet (Treatments 4-6; 33.3%, 70.0%, and 80.0%, respectively) and those fed Treatment 1 (Table 40).

SCR fed Treatments 3-6 had a significantly greater probability of reduced weight as compared to those fed Treatment 1, with no significant difference in probability observed for Treatment 2 (Table 41).

Materials and methods for bioassay analysis of SCR are presented in Volume 2, Appendix D, Section D2.

Table 40. Summary Analysis of SCR DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/ml)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	30	3	10.0	--
2	Test Diet	1	30	4	13.3	0.5000
3	Test Diet	10	30	3	10.0	0.6646
4	Test Diet	100	30	10	33.3	0.0287[a]
5	Test Diet	1000	30	21	70.0	<0.0001[a]
6	Test Diet	10000	30	24	80.0	<0.0001[a]

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatments 2-6 was based on diet wet weight.
[a] A statistically significant difference (P-value < 0.05) was observed.

Table 41. Summary Analysis of SCR DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/ml)	Number of Surviving Organisms	Mean Standard Deviation (mg)	Median Weight (mg)	Range (mg)	Wilcoxon Test P-Value	Siegel Tukey Test P-Value
1	Bioassay Control Diet	0	27	7.94 ± 6.34	5.1	0.4 - 22.6	--	--
2	Test Diet	1	26	8.01 ± 7.42	5.05	0.4 - 20.8	0.3029	0.0494[a]
3	Test Diet	10	27	3.69 ± 3.55	2.7	0.4 - 15.0	0.0031[a]	0.9862
4	Test Diet	100	20	1.49 ± 1.07	1.2	0.3 - 3.7	<0.0001[a]	0.7354
5	Test Diet	1000	9	3.30 ± 2.39	2.5	0.2 - 8.2	0.0163[a]	0.8693
6	Test Diet	10000	6	2.03 ± 1.81	1.6	0.4 - 4.6	0.0062[a]	0.4589

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatments 2-6 was based on diet wet weight.
[a] A statistically significant difference (P-value < 0.05) was observed.

IX-B.2.b.3. Colorado Potato Beetle (CPB)

The objective of this bioassay was to evaluate the survival and weight of CPB when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). CPB was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

CPB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 5000 ng cryolite per mg diet dry weight)

Each diet was provided to 30 individual CPB for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed every other day. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between CPB provided Treatment 1 and Treatment 2.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity and stability under bioassay conditions of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. A sensitive insect bioassay demonstrated the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of CPB (Table 42 and Table 43). Methods and materials for the bioassay evaluation of CPB are presented in Volume 2, Appendix D, SectionD3.

Table 42. Summary Analysis of CPB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	29 [a]	1	3.45	--
2	Test Diet	1	30	1	3.33	0.7627
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Wells that contained more than one organism were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 5000 ng cryolite per mg diet dry weight.

Table 43. Summary Analysis of CPB DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	136 (118 - 154)	7.2 - 217.0	--
2	Test Diet	1	29	153 (141 - 165)	54.7 - 200.8	0.9471
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving CPB in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 5000 ng cryolite per mg diet dry weight.

IX-B.2.b.4. Yellow Mealworm (MWM)

The objective of this bioassay was to evaluate the survival and weight of MWM when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). MWM was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

MWM larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weig ht)

Each diet was provided to 30 individual MWM for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour continuous dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between MWM provided Treatment 1 and Treatment 2.

The results demonstrated the mortality for MWM fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 23.3%) was not significantly greater than the mortality for MWM fed the bioassay control diet (Treatment 1; 13.3%) (Table 44). The mean weight of MWM fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (0.843 mg) was not significantly less than the mean weight of MWM fed Treatment 1 (0.923 mg) (Table 45).

Methods and materials for the bioassay evaluation of MWM are presented in Volume 2, Appendix D, Section D4.

Table 44. Summary Analysis of MWM DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	4	13.3	-
2	Test Diet	1	30	7	23.3	0.2531
3	Positive Control Diet	0[a]	30	18	60.0	-

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

Table 45. Summary Analysis of MWM DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	26	0.923 (0.808 - 1.04)	0.3 - 1.5	-
2	Test Diet	1	23	0.843 (0.721 - 0.966)	0.3 - 1.6	0.1673
3	Positive Control Diet	0[a]	12	0.483 ± 0.119[b]	0.4 - 0.8	-

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.
[b] Standard deviation is provided for mean values not subjected to a *t*-test.

IX-B.2.b.5. Superworm (SWM)

The objective of this bioassay was to evaluate the survival and weight of SWM when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). SWM was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

SWM larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weig ht)

Each diet was provided to 30 individual SWM for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between SWM provided Treatment 1 and Treatment 2. Methods and materials for the bioassay evaluation of SWM are presented in Volume 2, Appendix D, Section D6.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of SWM (Table 46 and Table 47).

Table 46. Summary Analysis of SWM DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	2	6.67	--
2	Test Diet	1	29[a]	2	6.90	0.6811
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

Table 47. Summary Analysis of SWM DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	1.20 (1.07 - 1.33)	0.8 - 2.0	--
2	Test Diet	1	27	1.21 (1.04 - 1.39)	0.5 - 2.3	0.5547
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving SWM in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

IX-B.2.b.6. Red Flour Beetle (RFB)

The objective of this bioassay was to evaluate the survival and weight of RFB when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). RFB was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

RFB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 3000 ng boric acid per mg diet dry weight)

Three independent bioassays were conducted. Each diet was provided to 30 individual RFB per bioassay for a total of 14 days. The bioassays were conducted in an environmental chamber set at 30 °C, 70% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After 14 days, each bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between RFB provided Treatment 1 and Treatment 2.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in each test dosing solution, the homogeneity of DvSSJ1_210 dsRNA in each test diet (Treatment 2), and the stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions and frozen storage. The absence of DvSSJ1_210 dsRNA in each bioassay control diet (Treatment 1) was also verified. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in each Treatment 2. Methods and materials for the bioassay evaluation of RFB are presented in Volume 2, Appendix D, Section D7.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of RFB (Table 48 and Table 49).

Table 48. Summary Analysis of RFB DvSSJ1 dsRNA Bioassay Mortality Results

Bioassay	Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	1	Bioassay Control Diet	0	28[a]	0	0	--
	2	Test Diet	1	27[a]	4	14.8	0.0515
	3	Positive Control Diet	0[b]	28[a]	28	100	--
2	1	Bioassay Control Diet	0	30	0	0	--
	2	Test Diet	1	29[a]	0	0	1.0000
	3	Positive Control Diet	0[b]	29[a]	29	100	--
3	1	Bioassay Control Diet	0	30	1	3.33	--
	2	Test Diet	1	30	1	3.33	0.7542
	3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay or lost in transfer, or cups containing more than one organism, were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 3000 ng boric acid per mg diet dry weight.

Table 49. Summary Analysis of RFB DvSSJ1 dsRNA Bioassay Weight Results

Bioassay	Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	1	Bioassay Control Diet	0	28	2.00 (1.82 - 2.18)	1.0 - 2.7	--
	2	Test Diet	1	23	1.90 (1.61 - 2.19)	0.3 - 3.0	0.2738
	3	Positive Control Diet	0[a]	0	NA	NA	--
2	1	Bioassay Control Diet	0	30	2.45 (2.32 - 2.59)	1.5 - 3.1	--
	2	Test Diet	1	29	2.48 (2.35 - 2.61)	1.9 - 3.7	0.5973
	3	Positive Control Diet	0[a]	0	NA	NA	--
3	1	Bioassay Control Diet	0	29	2.41 (2.19 - 2.62)	0.1 - 3.3	--
	2	Test Diet	1	29	2.47 (2.30 - 2.64)	1.7 - 3.4	0.6877
	3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving *RFB* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 3000 ng boric acid per mg diet dry weight.

IX-B.2.b.7. Mexican Bean Beetle (MBB)

The objective of this bioassay was to evaluate the survival and weight of MBB when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). MBB was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

MBB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 1250 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual MBB for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between MBB provided Treatment 1 and those provided Treatment 2.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in the test diet (Treatment 2). In addition, stability under bioassay conditions of DvSSJ1_210 dsRNA in Treatment 2 was assessed. The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2. Methods and materials for the bioassay evaluation of MBB are presented in Volume 2, Appendix D, Section D8.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of MBB (Table 50 and Table 51).

Table 50. Summary Analysis of MBB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	29[a]	0	0	--
2	Test Diet	1	30	0	0	1.0000
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 1250 ng boric acid per mg diet wet weight.

Table 51. Summary Analysis of MBB DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	29	3.22 (2.90 - 3.55)	1.7 - 5.2	--
2	Test Diet	1	30	3.11 (2.89 - 3.33)	2.3 - 5.0	0.2693
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving *MBB* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 1250 ng boric acid per mg diet wet weight.

IX-B.2.b.8. Convergent Lady Beetle (CNV)

The objective of this bioassay was to evaluate the survival, weight, and development of CNV when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). CNV was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

CNV larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 15,000 ng boric acid per mg diet dry weight)

Each diet was provided to 30 individual CNV until the time of pupation. The 28-day bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 17, all organisms had either died or emerged and the bioassay was complete. Mortality, weight, and number of days to adult emergence were statistically compared between CNV provided Treatment 1 and those provided Treatment 2.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival, weight, or adult emergence of CNV (Table 52, Table 53, and Table 54).

Methods and materials for the bioassay evaluation of CNV are presented in Volume 2, Appendix D, Section D9.

Table 52. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	2	6.67	--
2	Test Diet	1	27[a]	2	7.41	0.6531
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 53. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	19.1 (17.8 - 20.3)	14.3 - 26.2	--
2	Test Diet	1	25	18.0 (16.7 - 19.3)	12.8 - 24.5	0.1157
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 54. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Results for Days to Adult Emergence

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Data Points	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel Tukey Test P-Value
1	Bioassay Control Diet	0	28	15.1 ± 0.786	15	13 - 17	--	--
2	Test Diet	1	25	15.2 ± 0.879	15	14 - 17	0.3972	0.6016
3	Positive Control Diet	0[a]	0	NA	NA	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving *CNV* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

IX-B.2.b.9. Pink Spotted Lady Beetle (CMAC)

The objective of this bioassay was to evaluate the survival and development of CMAC when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). CMAC was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

CMAC larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 10,000 ng cryolite per mg diet dry weight)

Each diet was provided to 30 individual CMAC until the time of pupation. The 28-day bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 20, all organisms had either died or emerged and the bioassay was complete. Mortality, weight, and number of days to adult emergence were statistically compared between CMAC provided Treatment 1 and those provided Treatment 2.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity, frozen storage stability, and stability under bioassay conditions of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2. Methods and materials for the bioassay evaluation of CMAC are presented in Volume 2, Appendix D, Section D11.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival, weight, or adult emergence of CMAC (Table 55, Table 56, and Table 57).

Methods and materials for the bioassay evaluation of CMAC are presented in Volume 2, Appendix D, Section D13.

Table 55. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	1	29[a]	3	10.3	0.1124
3	Positive Control Diet	0[b]	28[a]	28	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

Table 56. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	12.6 (11.7 - 13.5)	7.8 - 18.0	--
2	Test Diet	1	26	13.0 (12.3 - 13.7)	9.2 - 16.8	0.7611
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CMAC in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

Table 57. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Days to Adult Emergence Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	30	15.0 ± 1.17	15	14 - 20	--	--
2	Test Diet	1	26	14.6 ± 0.983	14	13 - 17	0.9429	0.2679
3	Positive Control Diet	0[a]	0	NA	NA	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CMAC in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

IX-B.2.b.10. Rove Beetle (RVB)

The objective of this bioassay was to evaluate the survival of RVB when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). RVB was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

RVB adults were exposed via oral ingestion to one of the following three treatments

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 20,000 ng boric acid per mg diet dry weigh t)

Each diet was provided to 30 individual RVB for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Adults were refed daily. After 14 days, the bioassay was complete and mortality was assessed. Mortality was statistically compared between RVB provided Treatment 1 and those provided Treatment 2. Methods and materials for the bioassay evaluation of RVB are presented in Volume 2, Appendix D, Section D12.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival of RVB (Table 58).

Methods and materials for the bioassay evaluation of RVB are presented in Volume 2, Appendix D, Section D13.

Table 58. Summary Analysis of RVB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	1	30	0	0	1.0000
3	Positive Control Diet	0 [a]	30	30	100	--

Note: Targeted DvSSJ1_210 dsRNA concentrations in Treatment 2 were based on diet dry weight.
[a] Treatment 3 contained a targeted concentration of 20,000 ng boric acid per mg diet dry weight.

IX-B.2.b.11. European Corn Borer (ECB)

The objective of this bioassay was to evaluate the survival and weight of ECB when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). ECB was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

ECB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 3750 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual ECB for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between ECB provided Treatment 1 and Treatment 2.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of ECB (Table 59 and Table 60).

Methods and materials for the bioassay evaluation of ECB are presented in Volume 2, Appendix D, Section D13.

Table 59. Summary Analysis of ECB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	29[a]	2	6.90	--
2	Test Diet	1	30	3	10.0	0.5165
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 3750 ng boric acid per mg diet wet weight.

Table 60. Summary Analysis of ECB DvSSJ1 dsRNA Bioassay ECB Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	27	98.2 (86.5 - 110)	7.7 - 141.9	--
2	Test Diet	1	27	107 (97.6 - 117)	33.7 - 153.1	0.8843
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving *ECB* in the positive control diet group.

[a] Treatment 3 contained a targeted concentration of 3750 ng boric acid per mg diet wet weight.

IX-B.2.b.12. Corn Ear Worm (CEW)

The objective of this bioassay was to evaluate the survival and weight of CEW when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). CEW was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

CEW larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 3125 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual CEW for a total of 12 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 12 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between CEW provided Treatment 1 and Treatment 2.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2) had no adverse effect on survival or weight of CEW (Table 61 and Table 62).

Methods and materials for the bioassay evaluation of CEW are presented in Volume 2, Appendix D, Section D14.

Table 61. Summary Analysis of CEW DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	26[a]	2	7.69	--
2	Test Diet	1	26[a]	2	7.69	0.6951
3	Positive Control Diet	0[b]	28[ac]	26	92.9	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] A transfer error killed four organisms each in Treatments 1 and 2 and one organism in Treatment 3. Those organisms were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 3125 ng boric acid per mg diet wet weight.

[c] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 62. Summary Analysis CEW DvSSJ1 dsRNA Bioassay of Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	24	408 (363 - 453)	45.9 - 573.0	--
2	Test Diet	1	24	396 (362 - 431)	81.2 - 513.6	0.3372
3	Positive Control Diet	0[a]	2	0.750 ± 0.636[b]	0.3 - 1.2	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Treatment 3 contained a targeted concentration of 3125 ng boric acid per mg diet wet weight.
[b] Standard deviation is provided for mean values not subjected to a *t*-test.

IX-B.2.b.13. Painted Lady (PL)

The objective of this bioassay was to evaluate the survival and weight of PL when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). PL was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

PL larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 2500 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual PL for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between PL provided Treatment 1 and Treatment 2.

The results demonstrated the mortality for PL fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 26.7%) was not significantly greater than the mortality for PL fed the bioassay control diet (Treatment 1; 20.0%). The mean weight of PL fed Treatment 2 (405 mg) was not significantly less than the mean weight of PL fed Treatment 1 (391 mg) (Table 63 and Table 64).

Methods and materials for the bioassay evaluation of PL are presented in Volume 2, Appendix D, Section D15.

Table 63. Summary Analysis of PL DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	6	20.0	--
2	Test Diet	1	30	8	26.7	0.3805
3	Positive Control Diet	0[a]	28[b]	27	96.4	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Treatment 3 contained a targeted concentration of 2500 ng boric acid per mg diet wet weight.
[b] Due to transfer error, two organisms were not included in mortality calculations for Treatment 3.

Table 64. Summary Analysis of PL DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	24	391 (336 - 445)	86.7 - 566.4	--
2	Test Diet	1	22	405 (364 - 445)	226.7 - 565.3	0.6635
3	Positive Control Diet	0[a]	1	1.40	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there was only one surviving PL in the positive control diet group.
[a] Treatment 3 contained a targeted concentration of 2500 ng boric acid per mg diet wet weight.

IX-B.2.b.14. Codling Moth (CDM)

The objective of this study was to evaluate the survival and weight of CDM when exposed to DvSSJ1_210 double-stranded RNA (dsRNA). CDM was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA.

CDM larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 5000 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual CDM for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between CDM provided Treatment 1 and Treatment 2.

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2) had no adverse effect on survival or weight of CDM (Table 65 and Table 66).

Methods and materials for the bioassay evaluation of CDM are presented in Volume 2, Appendix D, Section D16.

Table 65. Summary Analysis of CDM DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	26[a]	3	11.5	--
2	Test Diet	1	25[a]	2	8.00	0.8129
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 5000 ng boric acid per mg diet wet weight.

Table 66. Summary Analysis of CDM DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	23	56.5 (50.3 - 62.6)	20.8 - 82.9	--
2	Test Diet	1	23	54.2 (48.0 - 60.5)	3.4 - 77.2	0.2979
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving CDM in the positive control diet group.

[a] Treatment 3 contained a targeted concentration of 5000 ng boric acid per mg diet wet weight.

IX-B.3. DvSSJ1 dsRNA Specificity Conclusions

As stated previously, when DP23211 maize plants expressing DvSSJ1 dsRNA are ingested by WCR, production of the DvSSJ1 protein in the intestinal lining is suppressed. Reduction in the DvSSJ1 protein, and the subsequent loss of the gut epithelial barrier and cellular deformities, is lethal to WCR (Hu et al., 2016; Hu et al., 2019). Since the mode of action of DvSSJ1 ds RNA is sequence based, bioinformatics analyses were combined with spectrum of activity insect bioassays for DvSSJ1 dsRNA to support the conclusion that the activity of DvSSJ1 dsRNA is limited to the genus *Diabrotica*.

The closest sequence match (percent identity to the 210-bp *dvssj1* sequence) was the *ssj1* homologous gene from WCR, which as intended had a 100% sequence match, 0 SNPs, and 190 21-nt matches (Table 38 and summarized in Table 67). The *ssj1* homologous gene from the closely

related species, NCR, shared 97.1% identity with the 210-bp *dvssj1* sequence, with 6 SNPs and 135 21-nt matches. The *ssj1* homologous gene from SCR shared 92.9% identity with the 210-bp *dvssj1* sequence, with 15 SNPs and 79 21-nt matches. The *ssj1* homologous genes from the other Coleoptera within the family Chrysomelidae (CFB, SFB, and CPB) as well as species within the family Tenebrionidae, the family Coccinellidae, and the family Staphylinidae had decreasing percent identity with the 210-bp *dvssj1* sequence, ranging from 77.6 to 61.9% similarity and an increasing number of SNPs (ranging from 47 to 80). All Lepidoptera species within the four families also had lower percent identity with the 210-bp *dvssj1* sequence, ranging from 68.1 to 60% similarity, and an increased number of SNPs (ranging from 67 to 84). There were zero of 21-nt matches observed across all of the non-*Diabrotica* species analyzed. The molecular target of DvSSJ1 dsRNA has been shown to be specific to the *Diabrotica* species within the Chrysomelidae family of Coleoptera. Additionally, bioinformatic sequence comparisons of DvSSJ1 dsRNA 21-nt siRNAs to mammalian, avian and fish transcriptomes indicate no 21-nt siRNA exact matches.

To assess the spectrum of activity of the DvSSJ1 dsRNA, ten species which represent four families within the order Coleoptera were assessed using laboratory bioassays. Spectrum testing was primarily focused within the order Coleoptera because DvSSJ1 dsRNA is targeted to match the sequence of the smooth septate junction protein 1 (*dvssj1*) gene from WCR (Hu et al., 2019). Within the family Chrysomelidae, species tested included: WCR, SCR, and CPB. Within the family Tenebrionidae, species tested included: RFB, SWM, and MWM. Within the family Staphylinidae, RVB was tested. Within the family Coccinellidae, species tested included: MBB, CMAC, and CNV Additionally, four species representing four families within the order Lepidoptera (PL, ECB, CDM, and CEW) were assessed to test for cross-order activity. For DvSSJ1 dsRNA, WCR was the most sensitive species tested, with an LC_{50} of 0.036 ng DvSSJ1 dsRNA/mg. The only other species showing sensitivity to DvSSJ1 dsRNA was SCR, which was observed to have decreased survival at concentration 0.1 µg DvSSJ1 dsRNA/ml diet (Table 40, Table 41, and summarized in Table 67).

Bioinformatics analyses were combined with the spectrum of activity data generated using insect bioassays for DvSSJ1 dsRNA and support the conclusion that that activity of DvSSJ1 dsRNA is limited to the genus *Diabrotica*. WCR was the most sensitive species tested in laboratory bioassays, and no adverse effects were noted in the Lepidoptera species. Therefore, based on the specificity of the DvSSJ1 dsRNA the ERA for DP23211 maize is primarily focused on non-target coleopterans.

Table 67. Summary of DvSSJ1 dsRNA Spectrum of Activity Results

Common Name	Species	Bioassay Results	Bioinformatic Results		
		NOEC (ng DvSSJ1 dsRNA/mg diet)	% DvSSJ1 similarity	# of SNPs	# of 21-nt matches
Western Corn Rootworm	*Diabrotica virgifera virgifera*	LC_{50} = 0.036 ng	100	0	190
Southern Corn Rootworm	*Diabrotica undecimpunctata*	100 (ng/ml)	92.9	15	79
Colorado Potato Beetle	*Leptinotarsa decemlineata*	1	73.3	56	0
Yellow Mealworm	*Tenebrio molitor*	1	65.2	73	0
Super Worm	*Zophobas morio*	1	69	65	0
Red Flour Beetle	*Tribolium castaneum*	1	69.5	64	0
Mexican Bean Beetle	*Epilachna varivestis*	1	67.6	68	0
Convergent Lady Beetle	*Hippodamia convergens*	1	-[a]	-[a]	-[a]
Pink Spotted Lady Beetle	*Coleomegilla maculata*	1	61.9	80	0
Rove Beetle	*Dalotia coriaria*	1	64.3	75	0
European Corn Borer	*Ostrinia nubilalis*	1	64.2	79	0
Corn Earworm	*Helicoverpa zea*	1	60	84	0
Painted Lady	*Vanessa cardui*	1	64.8	74	0 (8)
Codling Moth	*Cydia pomonella*	1	60.5	83	0 (8)

[a] Convergent Lady Beetle was not analyzed for bioinformatic similarity to DvSSJ1

IX-C. Analysis of IPD072Aa Protein Specificity

IX-C.1. IPD072Aa Mode of Action

The IPD072Aa protein, encoded by the *ipd072Aa* gene, provides control of corn rootworms (*Diabrotica spp*.) when expressed in plants. IPD072Aa is a non-pore forming protein that has a midgut site of action (SoA) where it targets and disrupts midgut epithelial cells causing breakdown of the epithelial lining. Specific binding of IPD072Aa to midgut epithelial cells was confirmed using competitive binding assays with brush border membrane vesicles (BBMVs) prepared from WCR midgut tissue. Binding of the IPD072Aa protein to WCR BBMVs did not occur under alkaline conditions, which mimic the lepidopteran midgut environment, and was also absent when using BBMVs prepared from ECB midgut tissue. These results are consistent with specific binding of the IPD072A protein in the WCR midgut being responsible for its insecticidal activity. Additionally, the lack of binding under alkaline conditions is consistent with no bioactivity of IPD072Aa protein against lepidopteran insects. The IPD072Aa protein will be shown to be specific in laboratory insect diet feeding studies, with activity limited to within the order Coleoptera (see Section IX-C.2. IPD072Aa Spectrum of Activity and Species Specificity).

The structure of the IPD072Aa is known (to be published, 2020) and functional equivalency data support the hypothesis that the folding of the IPD072Aa protein forms a dimer, creating a hydrophobic face. Functional equivalency data (to be published, 2020) also shows that the folding is equivalent between *in Planta* and bacterially produced IPD072Aa protein, which validates the use of bacterially produced IPD072Aa protein in artificial diet, laboratory based insect studies (used in studies listed in Section IX-C.2.a. Feeding Bioassay Assessment of IPD072Aa Protein Specificity). The IPD072Aa dimer is formed in the DP23211 maize plant, and it retains this form after ingestion until the protein enters the WCR midgut. Once in the midgut, the IPD072Aa protein dimer disassociates into a monomeric form which binds to midgut receptors. After binding, disruption of gut function caused by death of enterocytes leads to WCR death.

Data presented in subsequent sections below will show that IPD072Aa binds specifically to WCR BBMVs (Section IX-C.1.a. Specific Binding of the IPD072Aa Protein to Brush Border Membrane Vesicles), and IPD072Aa does not bind to the BBMVs of ECB (Section IX-C.1.b. Binding of the IPD072Aa Protein is absent from European Corn Borer Midgut Tissue). Cross-linking data presented below shows that the IPD072Aa protein dimer must dissociate to bind to its target receptor (Section IX-C.1.a. Specific Binding of the IPD072Aa Protein to Brush Border Membrane Vesicles). Immuno-detection using fluorescence-based confocal microscopy will show that the IPD072Aa protein is localized to the lumen of the WCR larval gut with intensification at enterocytes that line the gut (Section IX-C.1.c. IPD072Aa Protein Insect Mid-Gut Localization). Electron micrograph data will also show that the pathological effects of IPD072Aa protein are localized within the WCR gut and that larval mortality caused by IPD072Aa protein is due to disruption of gut function caused by death of enterocytes (Section IX-C.1.d Histopathology of Western Corn Rootworm Exposed to IPD072Aa Protein).

IX-C.1.a. Specific Binding of the IPD072Aa Protein to Brush Border Membrane Vesicles

To determine if the IPD072Aa protein could bind specifically to the cells that line the WCR midgut, BBMV binding was assessed. To monitor binding, IPD072Aa protein was covalently labeled with fluorescent dye, Alexa-Fluor488©, (referred to as Alexa-IPD072). In order to determine the conditions for optimal binding signal above background (i.e., specific binding), differing amounts of Alexa-IPD072 were incubated with differing amounts of BBMVs in the absence and presence of excess unlabeled IPD072Aa protein.

The binding of Alexa-IPD072 protein to BBMVs was monitored by using a digital imaging system to measure in-gel fluorescence of the protein following separation by SDS-PAGE. Incubation of WCR BBMVs (20 μg) with Alexa-IPD072 (20 nM) in the absence and presence of increasing concentrations of unlabeled IPD072Aa protein revealed specific binding of IPD072Aa protein that has a relatively low binding affinity, showing half displacement concentration value (EC_{50}) of Alexa-IPD072 at 714 ± 120 nM unlabeled protein (Figure 38). It should be noted that IPD072Aa

protein exists as a natural dimer in solution and the concentration for binding assumes binding of the monomeric form since protein concentrations were estimated by in-gel Coomassie staining using a BSA standard curve after separation by SDS-PAGE. Cross-linking of IPD072Aa protein to prevent dissociation of the dimer revealed decrease in the apparent binding affinity (i.e., increased EC_{50} value) which is consistent with the idea that binding of the monomeric form occurs in the WCR BBMVs.

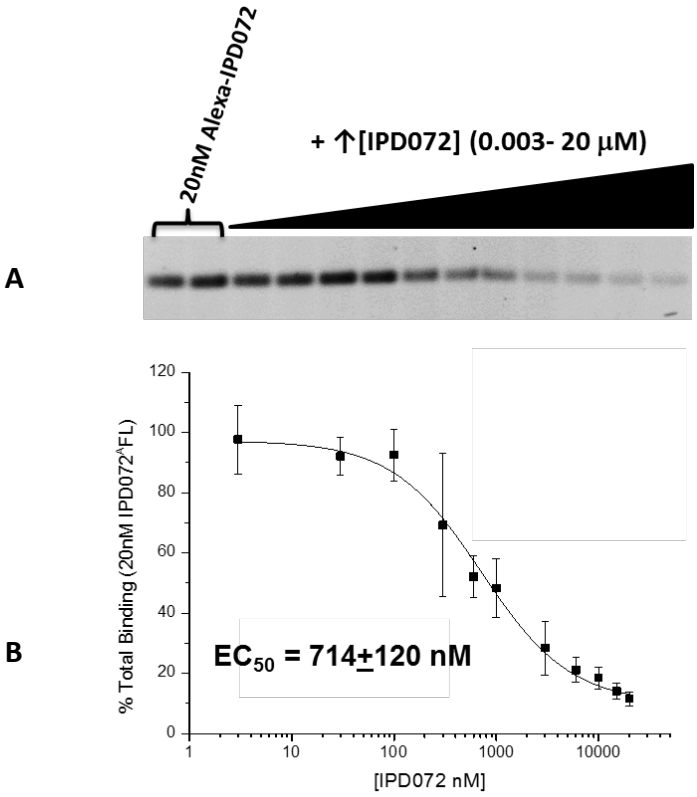


Figure 38. Specific binding of IPD072Aa to WCR BBMVs demonstrated by homologous competition.

A) A representative gel image showing the fluorescent signal remaining bound to WCR BBMVs as the concentration of unlabeled IPD072Aa protein was increased. B) Shows the averaged densitometry values along with the standard deviations from three binding experiments normalized to the signal in the absence of unlabeled IPD072Aa protein. The data were fit with a logistic equation to estimate the EC50 value which was 714 + 120 nM.

IX-C.1.b. Binding of the IPD072Aa Protein is absent from European Corn Borer Midgut Tissue

To better understand what might contribute to the spectrum of insecticidal activity of IPD072Aa, protein binding in WCR BBMVs was compared side-by-side to binding in BBMVs prepared from ECB midguts, one of several insect species where no bioactivity was observed when tested in artificial diet bioassays (Schellenberger et al., 2016). Specific binding was observed in WCR

BBMVs as shown by the ability of saturating concentrations of unlabeled IPD072Aa protein to displace the binding of Alexa-IPD072 (Figure 39 A), similar to the specific binding that appears in Figure 38. However, no binding was observed using ECB BBMVs (Figure 39 B). Because the lepidopteran midgut is very alkaline (Dow, 1992), while the WCR midgut is acidic (Murdock et al., 1987), the binding of the IPD072Aa protein was evaluated under alkaline conditions with WCR and ECB BBMVs in carbonate buffer, pH 9.6. No binding of Alexa-IPD072 was detected in either tissue, which indicates that alkaline conditions are not favorable for IPD072Aa protein binding. Overall, these results indicate that the IPD072Aa protein does not recognize a receptor target in ECB BBMVs and high pH is detrimental to IPD072Aa binding in WCR BBMV where a receptor target has been established.

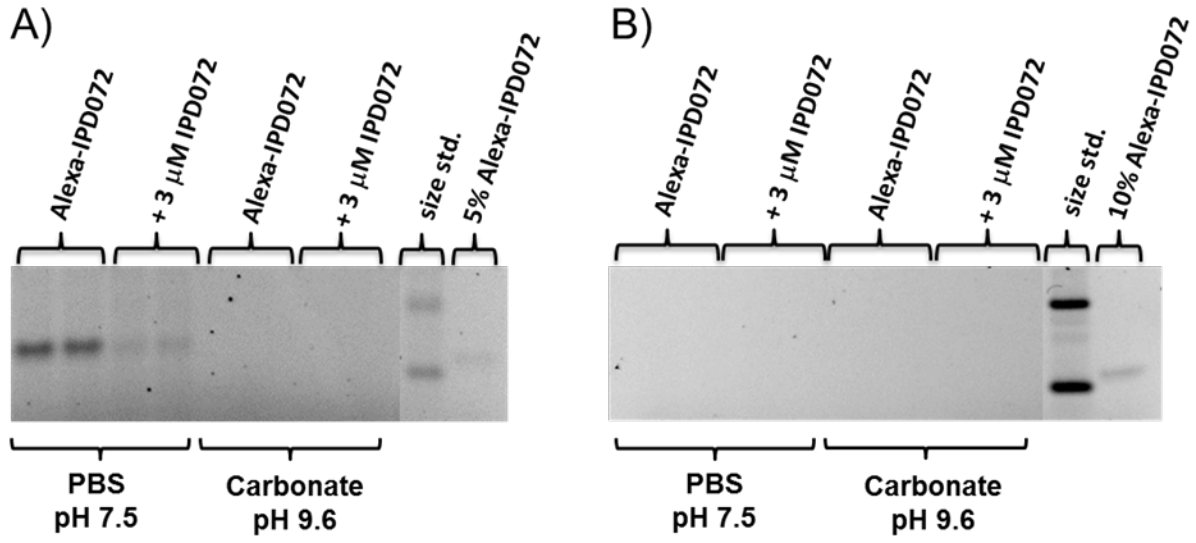


Figure 39. Specific binding of IPD072Aa protein to WCR BBMVs but not ECB BBMVs.
A) shows a representative gel image from competitive binding experiments using Alexa-IPD072 (50 nM) and WCR BBMVs (20 μg) where specific binding was observed using a phosphate buffered saline binding (PBS) buffer at near neutral pH, but not when using a carbonate buffer system at alkaline pH. B) shows a representative gel image from competitive binding experiments using Alexa-IPD072 (50 nM) and ECB BBMVs (20 μg) where no binding was observed when using either buffer system. Each gel image also includes a molecular weight size standard and a fixed amount of Alexa-IPD072 equivalent to a percentage of the amount needed for a 50 nM binding reaction.

IX-C.1.c. IPD072Aa Protein Insect Mid-Gut Localization

To evaluate the tissue localization and the histopathological effects of the IPD072Aa protein on WCR larvae, insects were allowed to feed on artificial diet that contained IPD072Aa protein at several doses (30, 60, and 90 ppm, equivalent to 30, 60, and 90 ng/mg) for various time points (12, 24, and 48 hrs). At each time point, larvae were collected, evaluated for feeding, and then subjected to chemical fixation to prepare for resin embedment to allow longitudinal sections

through the full length of a larva to be obtained. Immuno-detection using fluorescence-based confocal microscopy revealed the presence of IPD072Aa protein throughout the lumen of the larval gut with intensification at enterocytes that line the gut (Figure 40). The interaction of IPD072Aa protein with the enterocytes corroborates the finding of specific binding that was observed with BBMVs.

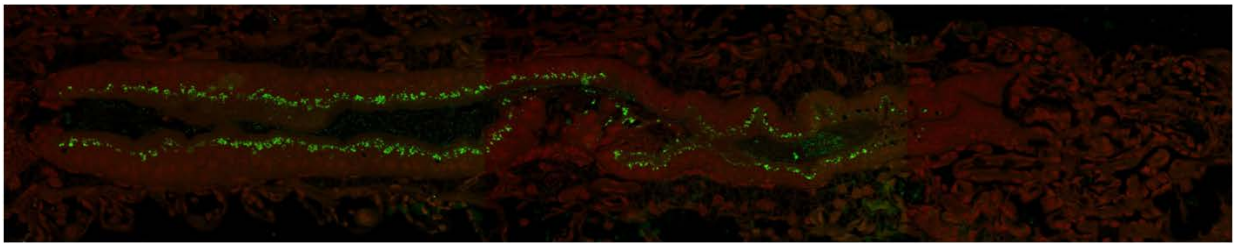


Figure 40. Immuno-Detection of IPD072Aa Protein in Sections of WCR Larvae Fed on Artificial Diet Containing IPD072Aa Protein.

The micrograph shows a longitudinal section through a WCR larva allowed to feed on IPD072Aa (60 ppm) for 12 hrs before processing for resin embedment to allow for sectioning (see Materials and Methods). Sections were then exposed to a primary Ab directed to IPD072Aa protein followed by an Alexa-488 labeled secondary antibody. The presence of IPD072Aa protein was detected by confocal microscopy and its distribution is represented by the green fluorescence contrasted by the natural red autofluorescence of WCR tissue.

IX-C.1.d Histopathology of Western Corn Rootworm Exposed to IPD072Aa Protein

WCR Larvae feeding on 60 ppm (ng/mg) IPD072Aa protein for 12 hrs exhibit slight changes in enterocyte morphology, mostly characterized by an apparent decrease in microvillar density or rigidity and increased prominence of low density organelles in the apical cytoplasm below the brush border membrane (Figure 41 A and Figure 41 B and C). With feeding on 90 ppm (ng/mg) IPD072Aa protein for 48 hrs, massive membrane blebbing and pinching-off of the apical membrane were observed. Remnants of enterocyte cytoplasm appear, some surrounded by plasma membrane and microvilli is throughout the lumen of gut (Figure 41 D). Dense dark vesicles are apparent at the apical plasma membrane, where blebbing is prominent, and in the membrane debris within the gut lumen (Figure 41 D). These observations show that the pathological effects of IPD072Aa protein are localized within the WCR gut and lead to the conclusion that larval mortality caused by IPD072Aa protein can be attributed to disruption of gut function caused by death of enterocytes.

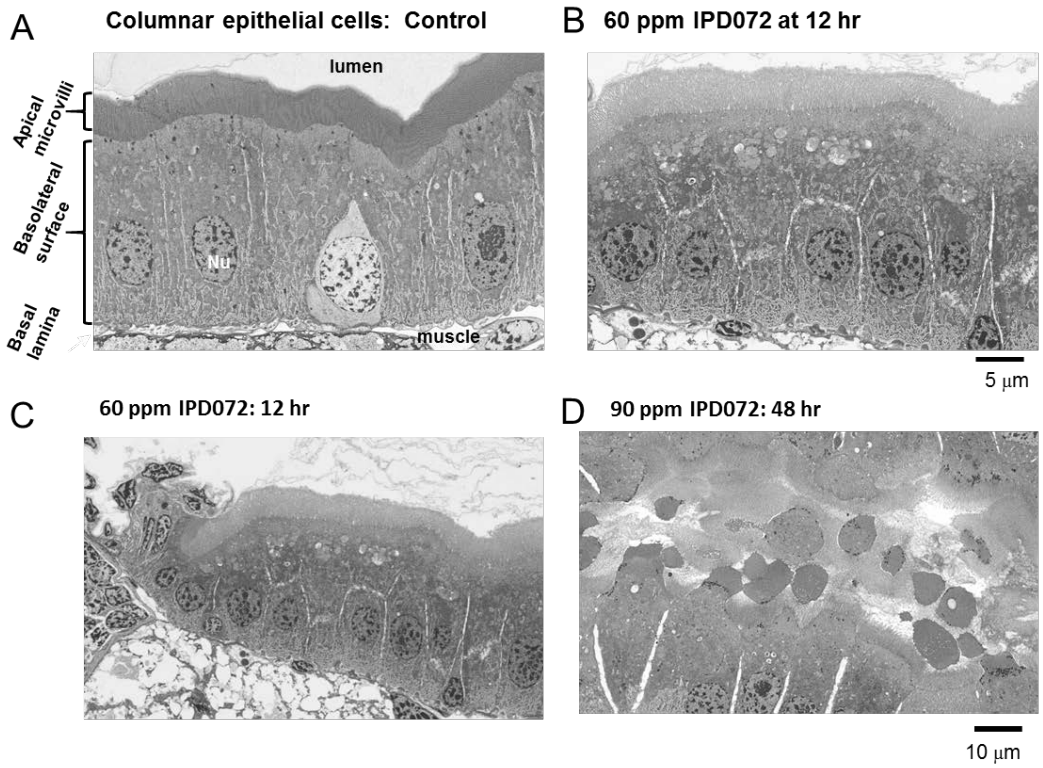


Figure 41. Electron micrographs showing the ultrastructure of WCR enterocytes after feeding on IPD072Aa protein.

A) The image depicts the appearance of WCR gut enterocytes of a larva that was fed on artificial diet only. A dense layer of microvilli is apparent at the apical surface just below the lumen of the gut. Other structural landmarks are labeled: the basolateral surface of the enterocytes, most of which are columnar epithelial cells; the basal lamina of the enterocytes where they incorporate into the basement membrane attaching to connective tissue and muscle surrounding the gut; and nuclei and muscle fibers are indicated. B) Shows the appearance of the enterocytes from a larva after feeding on 60 ppm (ng/mg) IPD072Aa protein for 12 hours. The microvilli begin to appear slightly less dense, reflecting the loss of structural rigidity. The presence of lower density organelles become more prominent. C) Shows a lower magnification of the same longitudinal section depicted in B) to provide perspective over a larger section of gut including the lumen and to give a sense of the uniformity of the effects of IPD072Aa protein. D) Depicts a longitudinal section at the same magnification as the section in C) showing the effects on a larva that fed on 90 ppm (ng/mg) IPD072Aa protein for 48 hrs. Extensive membrane blebbing and pinching-off of the apical membrane is apparent. The lumen of the gut is full of cellular debris. Dark vesicles are present along the plasma membrane, especially where the apical membrane appears to be preparing to separate. Dark vesicles are also present in the cellular debris within the lumen of the gut.

IX-C.1.e. Summary of IPD072Aa Mode of Action

The mode of action data presented above shows that IPD072Aa binds specifically to WCR BBMVs, and IPD072Aa does not bind to the BBMVs of ECB. Cross-linking data indicated that the IPD072Aa protein dimer must dissociate to bind to its target receptor. Immuno-detection using fluorescence-based confocal microscopy demostrated that the IPD072Aa protein is localized to the lumen of the WCR larval gut with intensification at enterocytes that line the gut. Electron

micrograph data also showed that the pathological effects of IPD072Aa protein are localized within the WCR gut and that larval mortality caused by IPD072Aa protein is due to disruption of gut function caused by death of enterocytes.

In summary, it can be concluded that the mode of action data presented here shows that the insecticidal mode of action of the IPD072Aa protein in DP23211 maize is through the disruption of mid-gut function that requires specific receptor-mediated binding of the IPD072Aa protein to the cells that line the midgut of WCR.

IX-C.2. IPD072Aa Spectrum of Activity and Species Specificity

The spectrum of activity of the IPD072Aa protein was assessed in feeding bioassays with WCR and 11 species from four families of Coleoptera (Chrysomelidae, Tenebrionidae, Coccinellidae, and Staphylinidae), and four species from four families of Lepidoptera (Crambidae, Tortricidae, Nymphalidae, and Noctuidae) (Volume 2, Appendix F).

IX-C.2.a. Feeding Bioassay Assessment of IPD072Aa Protein Specificity

Laboratory feeding bioassays were conducted on WCR, SCR, CPB, MWM, SWM, RFB, MBB, CNV, CMAC, MBD, RVB, ECB, CEW, PL, and CDM to determine the spectrum of activity of IPD072Aa protein. The objective of each bioassay feeding study was to evaluate the biological response of each test organism when fed an artificial diet containing IPD072Aa protein.

The bioassay methods, materials, and results are presented in the following sections and are also reviewed in Boeckman et.al., 2019.

IX-C.2.a.1. Western Corn Rootworm (WCR)

The objective of this study was to evaluate the biological response of WCR when fed artificial diet containing IPD072Aa protein. WCR is an insect sensitive to IPD072Aa protein.

Multiple independent dose-response bioassays were conducted exposing WCR larvae via oral ingestion to the following eight treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 2 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 4 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 8 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Test Diet (targeting 16 ng IPD072Aa protein per mg diet wet weight)
- Treatment 6: Test Diet (targeting 32 ng IPD072Aa protein per mg diet wet weight)
- Treatment 7: Test Diet (targeting 64 ng IPD072Aa protein per mg diet wet weight)
- Treatment 8: Test Diet (targeting 128 ng IPD072Aa protein per mg diet wet weight)

For each bioassay, each diet was provided to 30 individual WCR for a total of 7 days. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 3 or Day 4. After 7 days, the bioassays were complete, mortality was assessed, and surviving organisms were individually weighed. The dose-response relationship between the IPD072Aa protein concentration and *WCR* mortality was statistically analyzed for each bioassay and a median lethal concentration (LC_{50}) was estimated. An overall mean LC_{50} was estimated across the independent bioassays using a meta-analysis approach.

For each bioassay, a quantitative enzyme-linked immunosorbent assay (ELISA) method was used to verify the concentration of IPD072Aa protein in the working stock solutions used to prepare the dosing solutions for Treatments 2-8. The homogeneity of IPD072Aa protein in two diets per bioassay and the stability under bioassay conditions of Treatment 2 was also verified.

The estimated overall mean LC_{50} for WCR was 26 ng IPD072Aa protein per mg diet with a 95% confidence interval of 16-37 ng IPD072Aa protein per mg diet (Table 68).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F1.

Table 68. Median Lethal Concentration of IPD072Aa Protein for WCR

Bioassay	LC_{50}		
	Point Estimate	Standard Error	95% Confidence Interval
2	12	3.9	4.1 - 19
3	39	4.7	29 - 48
4	30	4.5	21 - 39
5	26	3.9	19 - 34
Overall Mean[a]	26	5.6	16 - 37

Note: Median lethal concentration (LC_{50}) is expressed in ng IPD072Aa protein per mg diet wet weight.
[a] The overall mean values as determined with the statistical analysis described in Volume 2, Appendix F, Section F1
Note: Standard error (SE).

VII-C.2.a.2. Southern Corn Rootworm (SCR)

The objective of this bioassay was to evaluate the survival and weight of SCR when exposed to IPD072Aa protein. SCR was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

SCR larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 10,000 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual SCR for a total of 7 days. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 4. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between SCR provided Treatment 1 and those provided Treatment 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatment 2 and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments

2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4.

The results demonstrated the mortality for SCR fed the test diets containing 100 ng IPD072Aa protein per mg diet (Treatment 2; 3.33%) and 500 ng IPD072Aa protein per mg diet (Treatment 3; 17.2%) was not significantly greater than the mortality for SCR fed the bioassay control diet (Treatment 1; 3.33%). A statistically significant difference was observed in mortality between SCR fed the test diet containing 1000 ng IPD072Aa protein per mg diet (Treatment 4; 25.0%) and those fed Treatment 1 (Table 69). In addition, statistically significant differences were observed in median weight between SCR fed Treatments 2, 3 and 4 (0.3 mg, 0.2 mg, and 0.2 mg, respectively) and those fed Treatment 1 (0.8 mg) (Table 70).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F Section F2.

Table 69. Summary Analysis of SCR IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	30	1	3.33	0.7542
3	Test Diet	500	29[a]	5	17.2	0.0896
4	Test Diet	1000	28[a]	7	25.0	0.0202[b]
5	Heat-treated Control Diet	1000	30	3	10.0	--
6	Positive Control Diet	0[c]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed.

[c] Treatment 6 contained a targeted concentration of 10,000 ng boric acid per mg diet wet weight.

Table 70. Summary Analysis of SCR IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Median (mg)	Mean Weight ± Standard Deviation (mg)	Range (mg)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	29	0.8	0.862 ± 0.235	0.5 - 1.4	--	--
2	Test Diet	100	29	0.3	0.300 ± 0.0926	0.2 - 0.6	<0.0001[a]	0.9387
3	Test Diet	500	24	0.2	0.192 ± 0.0584	0.1 - 0.3	<0.0001[a]	0.2509
4	Test Diet	1000	20[b]	0.2	0.170 ± 0.0733	0.1 - 0.4	<0.0001[a]	0.0546
5	Heat-treated Control Diet	1000	27	0.7	0.685 ± 0.123	0.3 - 0.9	--	--
6	Positive Control Diet	0[c]	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 10,000 ng boric acid per mg diet wet weight. Not applicable (NA); there were no surviving SCR in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] One organism was lost during weighing; this organism was not included in the total number of surviving organisms for weight results.

[c] Treatment 6 contained a targeted concentration of 10,000 ng boric acid per mg diet wet weight.

VII-C.2.a.3. Colorado Potato Beetle (CPB)

The objective of this bioassay was to evaluate the survival and weight of CPB when exposed to IPD072Aa protein. CPB was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

CPB larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa prot ein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 5000 ng cryolite per mg carrier dry weight)

Each diet was provided to 30 individual CPB for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 2, 4, and 6. After 7 days, the bioassay was complete, mortality was assessed,

and surviving organisms were individually weighed. Mortality and weight were statistically compared between CPB provided Treatment 1 and those provided Treatment 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatment 1 and Treatment 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4.

The results demonstrated exposure to a concentration of 100, 500, or 1000 ng IPD072Aa protein per mg diet (Treatments 2, 3, and 4, respectively) had no adverse effect on survival and weight of CPB (Table 71 and Table 72).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F Section F3.

Table 71. Summary Analysis of CPB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	0	0	1.0000
3	Test Diet	500	30	2	6.67	0.2458
4	Test Diet	1000	30	1	3.33	0.5000
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0	29[a]	29	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 5000 ng cryolite per mg carrier dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 72. Summary Analysis of CPB IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	24.6 (20.3 - 28.8)	0.8 - 48.8	--
2	Test Diet	100	30	20.8 (16.6 - 25.1)	1.0 – 59.0	0.1105
3	Test Diet	500	28	25.9 (21.5 - 30.2)	2.5 - 39.8	0.6645
4	Test Diet	1000	29	30.3 (26.0 - 34.6)	3.6 – 49.0	0.9683
5	Heat-treated Control Diet	1000	29	32.2 ± 14.7[a]	4.9 - 64.2	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 5000 ng cryolite per mg carrier dry weight. Not applicable (NA); there were no surviving CPB in the positive control diet group.
[a]Standard deviation is provided for mean values not subjected to a *t*-test.

VII-C.2.a.4. Mealworm (MWM)

The objective of this bioassay was to evaluate the survival and weight of MWM when exposed to IPD072Aa protein. MWM was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

MWM larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa prot ein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weig ht)

Each diet was provided to 30 individual MWM for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every three to four days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between MWM provided Treatment 1 and those provided Treatments 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4.

The results demonstrated the mortality for MWM fed the test diets containing 100 ng IPD072Aa protein per mg diet (Treatment 2; 20.0%) and 500 ng IPD072Aa protein per mg diet (Treatment 3; 33.3%) was not significantly greater than the mortality for MWM fed the bioassay control diet (Treatment 1; 13.3%). A statistically significant difference was observed in mortality between MWM fed the test diet containing 1000 ng IPD072Aa protein per mg diet (Treatment 4; 50.0%) and those fed Treatment 1 (Table 73). The mean weight for MWM fed Treatment 2 (0.913 mg) was not significantly less than the mean weight for MWM fed Treatment 1 (0.858 mg). Statistically significant differences were observed in mean weight between MWM fed Treatments 3 and 4 (0.665 mg and 0.580 mg, respectively) and those fed Treatment 1 (Table 74).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section 4.

Table 73. Summary Analysis of MWM IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	4	13.3	--
2	Test Diet	100	30	6	20.0	0.3653
3	Test Diet	500	30	10	33.3	0.0626
4	Test Diet	1000	30	15	50.0	0.0024[a]
5	Heat-treated Control Diet	1000	30	4	13.3	--
6	Positive Control Diet	0	30	29	96.7	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight. Treatment 6 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.
[a] A statistically significant difference (P-value < 0.05) was observed.

Table 74. Summary Analysis of MWM IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	26	0.858 (0.760 - 0.955)	0.4 - 1.5	--
2	Test Diet	100	24	0.913 (0.811 - 1.01)	0.5 - 1.5	0.7792
3	Test Diet	500	20	0.665 (0.554 - 0.776)	0.3 - 1.1	0.0057[a]
4	Test Diet	1000	15	0.580 (0.451 - 0.709)	0.4 - 1.1	0.0005[a]
5	Heat-treated Control Diet	1000	26	0.977 ± 0.208[b]	0.4 - 1.2	--
6	Positive Control Diet	0	1	0.400[c]	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 12,500 ng boric acid per mg diet wet weight. Not applicable (NA); there was only one surviving MWM in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Standard deviation is provided for mean values not subjected to a *t*-test.

[c]The reported mean is the weight value of the one surviving larva.

VII-C.2.a.5. Super Worm (SWM)

The objective of this bioassay was to evaluate the survival and weight of SWM when exposed to IPD072Aa protein. SWM was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

SWM larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa prot ein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weig ht)

Each diet was provided to 30 individual SWM for a total of 14 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between SWM provided Treatment 1 and those provided Treatments 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatment 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4.

The results demonstrated exposure to a concentration of 100 ng, 500 ng, or 1000 ng IPD072Aa protein per mg diet (Treatments 2, 3, and 4, respectively) had no adverse effect on survival of SWM. No statistically significant differences were observed in mortality between SWM fed Treatments 2, 3, and 4 (3.33%, 17.2%, and 20.0%, respectively) and those fed the bioassay control diet (Treatment 1; 3.33%) (Table 75). A statistically significant difference was observed in mean weight between SWM fed Treatments 2, 3, and 4 (0.886 mg, 0.921 mg, and 0.729 mg, respectively) and those fed Treatment 1 (1.11 mg) (Table 76).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F5.

Table 75. Summary Analysis of SWM IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	30	1	3.33	0.7542
3	Test Diet	500	29[a]	5	17.2	0.0896
4	Test Diet	1000	30	6	20.0	0.0514
5	Heat-treated Control Diet	1000	30	2	6.67	--
6	Positive Control Diet	0	29[a]	29	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight. Treatment 6 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

[a] Organisms counted as missing during the bioassay, or wells containing more than one organism, were not included in the total number of observations for a given treatment.

Table 76. Summary Analysis of SWM IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	29	1.11 (1.00 - 1.22)	0.7 - 2.1	--
2	Test Diet	100	28	0.886 (0.773 - 0.998)	0.4 - 1.6	0.0025[a]
3	Test Diet	500	24	0.921 (0.799 - 1.04)	0.4 - 1.4	0.0109[a]
4	Test Diet	1000	24	0.729 (0.608 - 0.851)	0.4 - 1.2	<0.0001[a]
5	Heat-treated Control Diet	1000	28	1.20 ± 0.340[b]	0.5 - 2.2	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 12,500 ng boric acid per mg diet wet weight. Not applicable (NA); there were no surviving *SWM* in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Standard deviation is provided for mean values not subjected to Fisher's exact test.

VII-C.2.a.6. Red Flour Beetle (RFB)

The objective of this bioassay was to evaluate the survival and weight of RFB when exposed to IPD072Aa protein. RFB was selected as a representative coleopteran to characterize the spectrum of activity of purified IPD072Aa protein.

RFB larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa prot ein per mg diet dry weight)
- Treatment 4: Positive Control Diet (targeting 10,000 ng boric acid per mg diet dry weigh t)

Each diet was provided to 30 individual RFB for a total of 7 days. The bioassay was conducted in an environmental chamber set at 30 °C, 70% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3 and missing and dead larvae were recorded. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between RFB provided Treatments 1 and 2.

Western blot analysis was used to visually confirm the dose, homogeneity, freezer stability, and stability under bioassay conditions of the IPD072Aa protein in the test diet (Treatment 2). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control diet (Treatment 1) and heat-treated control diet (Treatment 3) was also assessed. A sensitive insect

bioassay was used to demonstrate the biological activity of the IPD072Aa protein used in the test diet (Treatment 2).

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on survival and weight of RFB. (Table 77 and Table 78)

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F6.

Table 77. Summary Analysis of RFB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	28[a]	1	3.57	0.7545
2	Test Diet	28[a]	1	3.57	
3	Heat-treated Control Diet	29[a]	0	0	--
4	Positive Control Diet	29[a]	29	100	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet dry weight.
[a]Organisms counted as missing during the bioassay, or cups containing more than one organism, were not included in the total number of observations for a given treatment.

Table 78. Summary Analysis of RFB IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean ± Standard Deviation (mg)	Range (mg)	Wilcoxon P-Value
1	Bioassay Control Diet	27	0.370 ± 0.179	0.1 - 0.9	0.0881
2	Test Diet	27	0.304 ± 0.143	0.1 - 0.7	
3	Heat-treated Control Diet	29	0.376 ± 0.143	0.1 - 0.6	--
4	Positive Control Diet	0	NA	NA	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet dry weight.

VII-C.2.a.7. Mexican Bean Beetle (MBB)

The objective of this bioassay was to evaluate the survival and weight of MBB when exposed to IPD072Aa protein. MBB was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

MBB larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 1250 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual MBB for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and/or weight were statistically compared between MBB provided Treatment 1 and those provided Treatments 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2 and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4.

The results demonstrated exposure to a concentration of 100 ng IPD072Aa protein per mg diet (Treatment 2) had no adverse effect on survival of MBB. No statistically significant differences were observed in mortality between MBB fed Treatment 2 (16.7%) and those fed the bioassay control diet (Treatment 1; 3.33%). A statistically significant difference was observed in mean weight between MBB fed Treatment 2 (0.436 mg) and those fed Treatment 1 (1.23 mg) (Table 80). Statistically significant differences in mortality were observed between MBB fed diets containing 500 ng/mg or 1000 ng/mg IPD072Aa protein (Treatments 3 and 4, 96.6% and 100%, respectively) and those fed Treatment 1 (Table 79).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F7.

Table 79. Summary Analysis of MBB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	30	5	16.7	0.0973
3	Test Diet	500	29[a]	28	96.6	<0.0001[b]
4	Test Diet	1000	30	30	100	<0.0001[b]
5	Heat-treated Control Diet	1000	30	0	0	--
6	Positive Control Diet	0	30	30	100	--.

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight. Treatment 6 contained a targeted concentration of 1250 ng boric acid per mg diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed.

Table 80. Summary Analysis of MBB IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	29	1.23 (1.14 - 1.33)	0.7 - 1.9	--
2	Test Diet	100	25	0.436 (0.333 - 0.539)	0.2 - 0.8	<0.0001[a]
3	Test Diet	500	1	0.5[b]	NA	--
4	Test Diet	1000	0	NA	NA	--
5	Heat-treated Control Diet	1000	30	1.27 ± 0.325[c]	0.6 - 2.3	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 1250 ng boric acid per mg diet wet weight. Not applicable (NA); there were no surviving MBB in Treatment 4 or Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] The reported mean is the weight value of the one surviving larva.

[c]Standard deviation is provided for mean values not subjected to a *t*-test.

VII-C.2.a.8. Convergent lady beetle (CNV)

The objective of this bioassay was to evaluate the survival and development of CNV when exposed to IPD072Aa protein. CNV was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

CNV larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)

- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 15,000 ng boric acid per mg diet dry weight)

Each diet was provided to 30 individual CNV until the time of pupation. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 28, the bioassay was complete. Mortality, weight, and number of days to adult emergence were statistically compared between *CNV* provided Treatment 1 and those provided Treatments 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatment 2, 3, and 4 and the stability under bioassay conditions and the frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4.

The results demonstrated the mortality for CNV fed the test diets containing 100 ng and 500 ng IPD072Aa protein per mg diet (Treatment 2 and Treatment 3, respectively; both 0%) was not significantly greater than the mortality for CNV fed the bioassay control diet (Treatment 1; 0.0%). A statistically significant difference was observed in mortality between CNV fed the test diet containing 1000 ng IPD072Aa protein per mg diet (Treatment 4; 56.7%) and those fed Treatment 1 (Table 81). Statistically significant differences were observed in mean weight between CNV fed Treatments 2, 3 and 4 (18.6 mg, 11.3 mg, and 8.63 mg, respectively) and those fed Treatment 1 (19.7 mg) (Table 82). CNV fed Treatment 2 (median 14 days) and Treatment 1 (median 14 days) did not significantly differ in their probability to take longer to emerge. CNV fed Treatments 3 and 4 (median 17 and 22 days, respectively) had significantly greater probability to take longer to emerge than those fed Treatment 1 (Table 83).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F8.

Table 81. Summary Analysis of CNV IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	0	0	1.0000
3	Test Diet	500	30	0	0	1.0000
4	Test Diet	1000	30	17[a]	56.7	<0.0001[b]
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0[c]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight.
[a] Two organisms had not emerged as adults by Day 28 of the bioassay and were scored as dead.
[b] A statistically significant difference (P-value < 0.05) was observed.
[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 82. Summary Analysis of CNV IPD072Aa Protein Bioassay Adult Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	19.7 (18.9 - 20.4)	16.6 - 23.8	--
2	Test Diet	100	30	18.6 (17.9 - 19.3)	11.6 - 22.6	0.0236[a]
3	Test Diet	500	30	11.3 (10.6 - 12.1)	8.9 - 15.2	<0.0001[a]
4	Test Diet	1000	13	8.63 (7.51 - 9.75)	6.9 - 11.7	<0.0001[a]
5	Heat-treated Control Diet	1000	29	18.5 ± 3.63[b]	11.5 - 25.1	--
6	Positive Control Diet	0[c]	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 6.
[a] A statistically significant difference (P-value < 0.05) was observed.
[b] Standard deviation is provided for mean values not subjected to a t-test.
[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 83. Summary Analysis of CNV IPD072Aa Protein Bioassay Results for Days to Adult Emergence

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	30	13.8 ± 0.664	14	13 - 15	--	--
2	Test Diet	100	30	14.1 ± 0.712	14	13 - 16	0.0551	0.7594
3	Test Diet	500	30	17.4 ± 1.63	17	15 - 21	<0.0001[a]	1.0000
4	Test Diet	1000	13	22.3 ± 2.25	22	19 - 25	<0.0001[a]	0.0015[a]
5	Heat-treated Control Diet	1000	29	14.6 ± 1.50	14	13 - 20	--	--
6	Positive Control Diet	0[b]	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving *CNV* in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

VII-C.2.a.9. Pink Spotted Lady Beetle (CMAC)

The objective of this bioassay was to evaluate the survival and development of CMAC when exposed to IPD072Aa protein. CMAC was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

CMAC larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 10,000 ng cryolite per mg diet dry weight)

Each diet was provided to 30 individual CMAC until the time of pupation. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photometric cycle of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 23, all organisms had either died or

emerged and the bioassay was complete. Mortality, weight, and number of days to adult emergence were statistically compared between CMAC provided Treatment 1 and those provided Treatments 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions and the storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4.

The results demonstrated the mortality for CMAC fed the test diet containing 100 ng IPD072Aa protein per mg diet (Treatment 2; 6.67%) was not significantly greater than the mortality for CMAC fed the bioassay control diet (Treatment 1; 6.90%). Statistically significant differences were observed in mortality between CMAC fed the test diets containing 500 ng and 1000 ng IPD072Aa protein per mg diet (Treatment 3 and 4; 26.7% and 36.7%, respectively) and those fed Treatment 1 (6.90%) (Table 84). The mean weight for CMAC fed Treatment 2 (12.1 mg) was not significantly less than the mean weight for CMAC fed Treatment 1 (12.0 mg). Statistically significant differences were observed in mean weight between CMAC fed Treatments 3 and 4 (10.7 mg and 10.4 mg, respectively) and those fed Treatment 1 (12.0 mg) (Table 85). CMAC fed Treatment 2 (median 15 days) and Treatment 1 (median 14 days) did not significantly differ in their probability to take longer to emerge. CMAC fed Treatments 3 and 4 (median 16 days) had significantly greater probability to emerge later than those fed Treatment 1 (Table 86).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F9

Table 84. Summary Analysis of CMAC IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	29[a]	2	6.90	--
2	Test Diet	100	30	2	6.67	0.7070
3	Test Diet	500	30	8	26.7	0.0449[b]
4	Test Diet	1000	30	11	36.7	0.0061[b]
5	Heat-treated Control Diet	1000	29[a]	3	10.3	--
6	Positive Control Diet	0	30	30	100	--.

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight. Treatment 6 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

Table 85. Summary Analysis of CMAC IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	27	12.0 (11.3 - 12.7)	5.4 - 14.9	--
2	Test Diet	100	28	12.1 (11.4 - 12.8)	9.0 - 15.7	0.5424
3	Test Diet	500	22	10.7 (9.96 - 11.5)	6.1 - 14.1	0.0073[a]
4	Test Diet	1000	19	10.4 (9.53 - 11.2)	8.2 - 12.3	0.0014[a]
5	Heat-treated Control Diet	1000	26	11.3 ± 1.63[b]	9.6 - 15.0	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Treatment 6 contains a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

[a] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

[b] Standard deviation is provided for mean values not subjected to linear mixed model analysis.

Table 86. Summary Analysis of CMAC IPD072Aa Protein Bioassay Days to Adult Emergence Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	27	14.6 ± 1.95	14	11 - 22	--	--
2	Test Diet	100	28	14.7 ± 1.18	15	13 - 17	0.1529	0.6183
3	Test Diet	500	22	16.6 ± 2.92	16	13 - 23	0.0015[a]	0.3759
4	Test Diet	1000	19	15.9 ± 2.05	16	13 - 20	0.0088[a]	0.1147
5	Heat-treated Control Diet	1000	26	14.3 ± 1.02	14.5	13 - 16	--	--
6	Positive Control Diet	0	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Treatment 6 contains a targeted concentration of 10,000 ng cryolite per mg diet dry weight. Not applicable (NA); there were no surviving *CMAC* in the positive control diet group.

[a] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

VII-C.2.a.10. Mealybug Destroyer (MBD)

The objective of this bioassay was to evaluate the survival and weight of MBD when exposed to IPD072Aa protein. MBD was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

MBD adults were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 37,500 ng boric acid per mg diet dry weight)

Each diet was provided to 30 individual MBD for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Adults were refed every 3 days. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between MBD provided Treatment 1 and those provided Treatments 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatment 2, 3, and 4 and the stability under bioassay conditions and the frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4.

No statistically significant differences in mortality were observed in MBD exposed to a concentration of 100, 500 or 1000 ng IPD072Aa protein per mg diet when compared to those fed the bioassay control diet. While survival differences were not statistically significant, MBD fed 100 and 1000 ng IPD072Aa protein per mg diet exceeded 20% mortality. Development (measured as weight gain over the 7-day bioassay) was statistically significantly lower in MBD fed 100 ng IPD072Aa protein per mg diet versus the bioassay control, but not with those fed 500 and 1000 ng IPD072Aa protein per mg diet. In the absence of a dose-dependent response, the statistically significant difference for 100 ng IPD072Aa protein per mg diet is not thought to be biologically relevant. It can be concluded that at concentrations up to 1000 ng IPD072Aa protein per mg diet, there was no effect on survival or weight gain of MBD (Table 87 and Table 88).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F10.

Table 87. Summary Analysis of MBD IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	6	20.0	--
2	Test Diet	100	30	8	26.7	0.3805
3	Test Diet	500	30	4	13.3	0.8505
4	Test Diet	1000	30	11	36.7	0.1258
5	Heat-treated Control Diet	1000	30	4	13.3	--
6	Positive Control Diet	0[a]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight.
[a] Treatment 6 contained a targeted concentration of 37,500 ng boric acid per mg diet dry weight.

Table 88. Summary Analysis of MBD IPD072Aa Protein Bioassay Weight Change Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Day 0		Day 7		P-Value
				Mean ± Standard Deviation (mg)	Range (mg)	Mean ± Standard Deviation (mg)	Range (mg)	
1	Bioassay Control Diet	0	24	9.38 ± 0.873	8.1 - 11.2	12.4 ± 1.17	10.7 - 16.0	--
2	Test Diet	100	22	9.66 ± 1.19	7.0 - 12.5	12.2 ± 1.20	10.0 - 14.1	0.0252 [a]
3	Test Diet	500	26	9.68 ± 0.809	8.3 - 11.3	12.5 ± 1.12	10.3 - 15.1	0.1667
4	Test Diet	1000	19	9.48 ± 0.741	8.0 - 10.8	12.5 ± 1.14	11.0 - 14.8	0.4681
5	Heat-treated Control Diet	1000	26	9.30 ± 1.03	7.4 - 11.7	12.0 ± 1.35	9.7 - 15.2	--
6	Positive Control Diet	0[b]	0	NA	NA	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight. Not applicable (NA); there were no surviving *Cryptolaemus montrouzieri* in Treatment 6.
[a] A statistically significant difference (P-value < 0.05) was observed.
[b] Treatment 6 contained a targeted concentration of 37,500 ng boric acid per mg diet dry weight.

VII-C.2.a.11. Rove beetle (RVB)

The objective of this bioassay was to evaluate the survival of RVB when exposed to IPD072Aa protein. RVB was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein.

RVB adults were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 37,500 ng boric acid per mg diet dry weight)

Each diet was provided to 30 individual RVB for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photometric cycle of 16 hours of light followed by 8 hours of dark. Adults were refed daily. After 7 days, the bioassay was complete and mortality was assessed. Mortality was statistically compared between RVB provided Treatment 1 and those provided Treatments 2, 3, or 4.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4.

The results demonstrated that exposure to a concentration of 100, 500, or 1000 ng IPD072Aa protein per mg diet (Treatments 2, 3, and 4, respectively) had no adverse effect on the survival of RVB (Table 89)

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F11.

Table 89. Summary Analysis of RVB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	1	3.33	0.5000
3	Test Diet	500	30	3	10.0	0.1186
4	Test Diet	1000	30	2	6.67	0.2458
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0	30	29	96.7	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight. Treatment 6 contained a targeted concentration of 37,500 ng boric acid per mg diet dry weight.

VII-C.2.a.12. European corn borer (ECB)

The objective of this bioassay was to evaluate the survival and weight of ECB when exposed to IPD072Aa protein. ECB was selected as a representative lepidopteran to characterize the spectrum of activity of purified IPD072Aa protein.

ECB larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (targeting 3750 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual ECB for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 4. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between ECB provided Treatments 1 and 2.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diet (Treatment 2). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control diet (Treatment 1) and heat-treated control diet (Treatment 3) was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in the test diet (Treatment 2).

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on survival or weight of ECB (Table 90 and Table 91).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section12.

Table 90. Summary Analysis of ECB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	30	2	6.67	1.0000
2	Test Diet	30	0	0	
3	Heat-treated Control Diet	30	0	0	--
4	Positive Control Diet	30	30	100	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

Table 91. Summary Analysis of ECB IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	*t*-Test P-Value
1	Bioassay Control Diet	28	6.70 (5.47 - 7.92)	0 - 12.7	0.5102
2	Test Diet	30	6.72 (5.64 - 7.79)	0.3 - 10.9	
3	Heat-Treated Control Diet	30	6.46 ± 2.34[a]	2.1 - 9.5	--
4	Positive Control Diet	0	NA	NA	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight. Not applicable (NA); there were no surviving *ECB* in the positive control diet group.
[a] Standard deviation is provided for mean values not subjected to a *t*-test.

VII-C.2.a.13. Corn Ear Worm (CEW)

The objective of this bioassay was to evaluate the survival and weight of CEW when exposed to IPD072Aa protein. CEW was selected as a representative lepidopteran to characterize the spectrum of activity of purified IPD072Aa protein.

CEW larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet targeting 1000 ng IPD072Aa protein per mg diet wet weight
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa prot ein per mg diet wet weight)
- Treatment 4: Positive Control Diet (containing boric acid)

Each diet was provided to 30 individual CEW for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 4 and missing and dead organisms were recorded. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between CEW provided Treatments 1 and 2.

A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in the test diet (Treatment 2). Western blot analysis was used to visually confirm the dose, homogeneity, and stability under bioassay conditions of the IPD072Aa protein in the test diet (Treatment 2). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control diet (Treatment 1) and heat-treated control diet (Treatment 3) was also assessed.

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on the survival or weight of CEW (Table 92 and Table 93).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F13.

Table 92. Summary Analysis of CEW IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	30	0	0	1.0000
2	Test Diet	30	0	0	
3	Heat-treated Control Diet	30	2	6.67	--
4	Positive Control Diet	30	14	46.7	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

Table 93. Summary Analysis of CEW IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	*t*-Test P-Value
1	Bioassay Control Diet	30	109 (98.4 - 119)	56.2 - 151.7	0.0704
2	Test Diet	30	97.5 (86.1 - 109)	11.6 - 162.4	
3	Heat-treated Control Diet	28	95.5 ± 42.3[a]	26.1 - 184.5	--
4	Positive Control Diet	16	0.325 ± 0.139[a]	0.1 - 0.6	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.
[a] Standard deviation is provided for mean values not subjected to a *t*-test.

VII-C.2.a.14. Painted Lady (PL)

The objective of this bioassay was to evaluate the survival and weight of PL when exposed to IPD072Aa protein. PL was selected as a representative lepidopteran to characterize the spectrum of activity of purified IPD072Aa protein.

PL larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (targeting 2500 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual PL for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between PL provided Treatments 1 and 2.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diet (Treatment 2). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control diet (Treatment 1) and heat-treated control diet (Treatment 3) and stability of the IPD072Aa protein dosing solution were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein used in the test diet (Treatment 2).

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on survival or weight of PL (Table 94 and Table 95)

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F14.

Table 94 Summary Analysis of PL IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	30	5	16.7	0.8729
2	Test Diet	30	3	10.0	
3	Heat-treated Control Diet	30	3	10.0	--
4	Positive Control Diet	30	26	86.7	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

Table 95. Summary Analysis of PL IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean ± Standard Deviation (mg)	Range (mg)	Wilcoxon Test P-Value
1	Bioassay Control Diet	25	18.2 ± 9.26	2.7 - 30.4	0.9138
2	Test Diet	27	21.9 ± 7.16	4 - 37.8	
3	Heat-Treated Control Diet	27	23.4 ± 6.68	5.8 - 30.9	--
4	Positive Control Diet	4	0.500 ± 0.141	0.4 - 0.7	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

VII-C.2.a.15. Codling Moth (CDM)

The objective of this bioassay was to evaluate the survival and weight of CDM when exposed to IPD072Aa protein. CDM was selected as a representative lepidopteran to characterize the spectrum of activity of purified IPD072Aa protein.

CDM larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (targeting 5000 ng boric acid per mg diet wet weight)

Each diet was provided to 30 individual CDM larvae for a total of 7 days. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between CDM provided Treatments 1 and 2.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diet (Treatment 2). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control diet (Treatment 1) and heat-treated control diet (Treatment 3) and stability of the IPD072Aa protein dosing solution were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in the test diet (Treatment 2).

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on mortality or weight of CDM (Table 96 and Table 97).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix F, Section F15.

Table 96. Summary Analysis of CDM IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	30	2	6.67	--
2	Test Diet	30	1	3.33	0.8814
3	Heat-treated Control Diet	29[a]	1	3.45	--
4	Positive Control Diet	30	30	100	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1,000 ng/mg based on diet wet weight.
[a] Organisms counted as missing during the bioassay, or wells that contained more than one organism, were not included in the total number of observations for a given treatment.

Table 97. Summary Analysis of CDM IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	t-Test P-Value
1	Bioassay Control Diet	28	5.65 (5.20 - 6.09)	2.8 - 7.6	--
2	Test Diet	29	5.58 (5.05 - 6.12)	1.7 - 8.1	0.4263
3	Heat-Treated Control Diet	28	5.44 ± 1.57[a]	1.6 - 7.9	--
4	Positive Control Diet	0	NA	NA	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1,000 ng/mg based on diet wet weight. Not applicable (NA); there were no surviving *CDM* in the positive control diet group.
[a] Standard deviation is provided for mean values not subjected to a *t*-test.

IX-C.3. IPD072Aa Protein Specificity Conclusions

To assess the spectrum of activity of the IPD072Aa protein, 11 species which represent 4 families within the order Coleoptera were assessed using laboratory bioassays. Spectrum testing was primarily focused within the order Coleoptera given the demonstrated activity of IPD072Aa on WCR (Schellenberger et al., 2016). Within the family Chrysomelidae, species tested included: WCR, SCR, and CPB. Within the family Tenebrionidae, species tested included: RFB, SWM, and MWM. Within the family Staphylinidae, RVB was tested. Within the family Coccinellidae, species tested included: MBB, MBD, CMAC, and CNV. Additionally, four species representing four families within the order Lepidoptera (PL, ECB, CDM, and CEW) were assessed to test for cross-order activity. For the IPD072Aa protein, WCR was the most sensitive species tested. There was a range

of responses observed within each of the four families of Coleoptera evaluated that included either no-observed effects or reduced growth, developmental delays, and/or reduced survival.

For the IPD072Aa protein, WCR was the most sensitive species tested, activity was only observed within the order Coleoptera, and no adverse effects to Lepidoptera species were noted. Therefore, based on the specificity of the IPD072Aa protein, the ERA for DP23211 maize is primarily focused on non-target coleopterans.

Table 98. Summary of IPD072Aa Protein Spectrum of Activity Results

Common Name	**No Observed Effect Concentration (ng/mg diet) or LC_{50}**	**Endpoints Measured**
Western Corn Rootworm	**26 ng/mg diet LC_{50}**	**Mortality**
Southern Corn Rootworm	500; 1000	Mortality; Weight
Colorado Potato Beetle	1000; 1000	Mortality; Weight
Mealworm	500; 500	Mortality; Weight
Super Worm	1000; 500	Mortality; Weight
Red Flour Beetle	1000; 1000	Mortality; Weight
Mexican Bean Beetle	100; Effects on weight at all doses	Mortality; Weight
Convergent Lady Beetle	500; Effects on weight at all doses; 500	Mortality; Weight;Emergence
Pink Spotted Lady Beetle	100; 100; 1000	Mortality; Weight; Emergence
Mealybug Destroyer	1000; 1000	Mortality; Weight
Rove Beetle	1000	Mortality
European Corn Borer	1000; 1000	Mortality; Weight
Corn Ear Worm	1000; 1000	Mortality; Weight
Painted Lady	1000; 1000	Mortality; Weight
Codling Moth	1000; 1000	Mortality; Weight

IX-D. Analysis of DP23211 Weediness Potential and NTO Exposure Assessment

IX-D.1. Potential for Outcrossing and Weediness

The potential for gene flow between maize and relatives of the genera *Zea* and *Tripsacum* is low. While wild native or introduced populations of these genera occur where maize is cultivated, limited geographic range and low fitness or sterility of hybrids prevent successful gene flow. Furthermore, none of these wild relatives are considered to be noxious weeds and DP23211 maize is not expected to exhibit greater potential for weediness. Therefore, any incidental gene flow between DP23211 maize and its wild relatives would not likely transform maize wild relatives into more weedy species, nor would the introduced trait be introgressed widely in wild relative populations.

EPA has previously determined that there is no significant risk of gene capture and expression of any *Bt* endotoxin by wild or weedy relatives in the United States, its possessions or territories (US-EPA, 2010a). Since these conclusions are based on the nature of pollination of maize, survival of maize hybrid offspring, and weediness in maize and its relatives, these conclusions should also apply to proteins derived from non-*Bt* sources, as well as dsRNA (US-EPA, 2015). Therefore, based

on what is known about the basic biology of maize, the receiving environment, and the intended traits, the risk of increased weediness or outcrossing is expected to be low.

IX-D.2. Non-Target Organism Exposure Assessment

The exposure assessment considers the probability and degree to which NTOs will be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein from DP23211 maize. Worst-case estimated environmental concentrations (EECs) of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize were determined using worst-case assumptions (i.e., maximum concentrations of DvSSJ1 dsRNA and the IPD072Aa protein in relevant DP23211 maize tissues) to determine potential exposure for NTOs, including pollinators and pollen feeders, soil-dwelling organisms, and predators and parasitoids. These representative functional groups of NTOs and their possible routes of exposure (i.e., relevant DP23211 maize tissues) are illustrated in Figure 42. Refined EECs were calculated under more realistic assumptions of environmental conditions, when there was an interest in understanding more environmentally relevant concentrations.

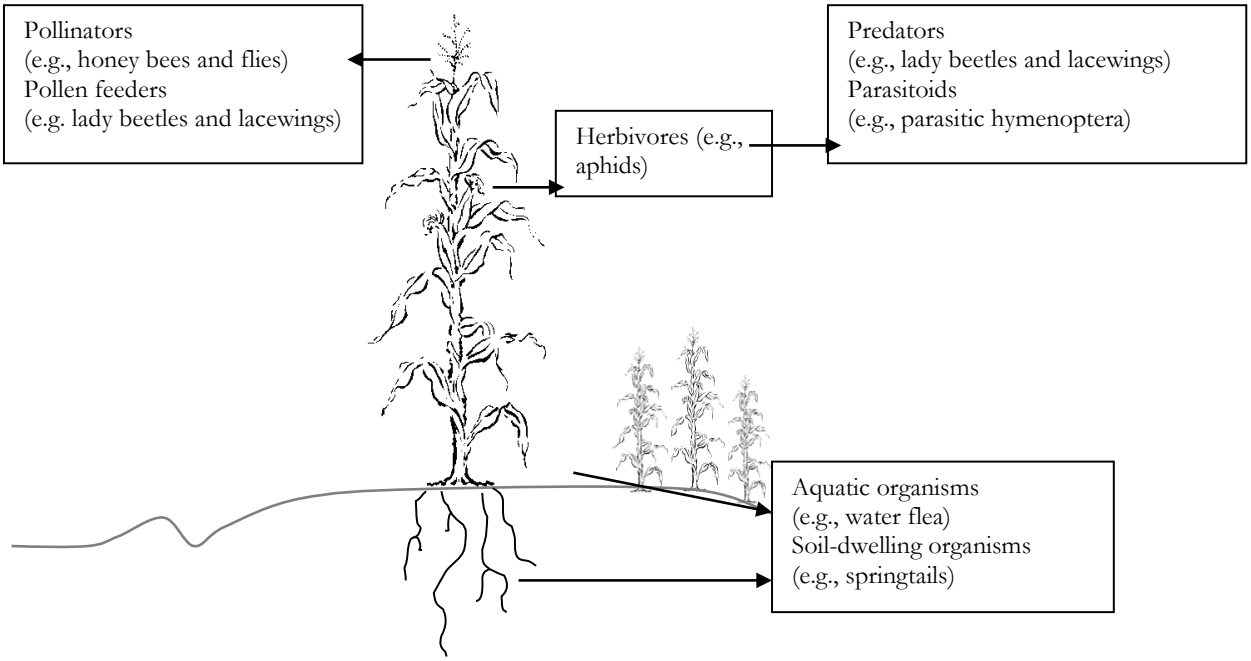


Figure 42. Representative Ecological Functional Groups and Possible Routes of Exposure

IX-D.2.a Pollinators and Pollen Feeders

To characterize risk, it is important to understand the potential for pollinators and pollen feeders to be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize. One key

consideration in the exposure assessment of pollen feeders and pollinators is the concentration of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen.

In a comprehensive field expression study, the mean concentration of the DvSSJ1 dsRNA was 9.87 x 10^{-4} ng/mg pollen (ranging from 5.61 x 10^{-4} – 2.02 x 10^{-3} ng/mg pollen dry weight (Table 18). The mean IPD072Aa protein concentration was 0.65 ng/mg pollen (ranging from 0.14 – 1.3 ng/mg pollen dry weight) (Table 19) for non-target pollinators and pollen feeders, the worst-case EEC assumes that pollen feeders and pollinators are exposed to the maximum concentration of DvSSJ1 dsRNA and the IPD072Aa protein in pollen (2.02 x 10^{-3} ng DvSSJ1 dsRNA/mg pollen or 1.3 ng IPD072Aa protein/mg pollen (Table 132 and Table 134).

For DP23211 maize, the maximum concentrations of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen are low, relative to the LC_{50} of the most sensitive target insect, WCR. The most sensitive target species tested is a useful indicator of potential effects on NTOs (US-EPA, 2001a). The LC_{50} of DvSSJ1 dsRNA to WCR is 0.036 ng/mg which is approximately 18 times higher than the maximum concentration of DvSSJ1 dsRNA in DP23211 maize pollen. The LC_{50} of the IPD072Aa protein to WCR is 26 ng/mg approximately 20 times higher than the concentration of the IPD072Aa protein in DP23211 maize pollen.

Based on the concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen, the worst-case exposure of non-target pollinators and pollen feeders to the DvSSJ1 dsRNA is considered negligible. Several additional factors will further reduce the actual exposure of non-target pollinators and pollen feeders to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs.

IX-D.2.a.1. Honey bees (*Apis mellifera*)

The potential exposure of honey bees to the DvSSJ1 dsRNA and IPD072Aa protein in DP23211 maize pollen is assessed due to their importance as beneficial organisms in agriculture and the agroecosystem. Based on the concentration of DvSSJ1 dsRNA and IPD072Aa protein in DP23211 maize pollen, the worst-case EEC of honey bees is considered negligible, as discussed above. Many factors will reduce actual exposure below the worst-case EECs, including the degree of spatial overlap of honey bee foraging ranges with DP23211 maize fields as well as the degree of temporal overlap of maize anthesis with sensitive insect life stages. Honey bee larvae are regarded as the most sensitive life stage for assessing the risk of cultivating DP23211 maize to honey bees.

Honey bees can be found in habitats where abundant supplies of flowering plants are present, and honey bees are common in agricultural fields. Honey bee foraging distances normally range from a few hundred yards up to 6 miles to collect pollen and nectar (Rose et al., 2007) and have been reported to range up to 8 miles when resources are scarce near the hive (Morse, 1972).

Because pollen is a primary food source, honey bees are likely to be present and actively foraging during pollen shed (Crailsheim et al., 1992). However, population level exposure to DP23211 maize pollen is expected to be low as maize pollen is not expected to be the only dietary component consumed. Adult honey bees feed on pollen from a variety of different plant species as a major source of protein and consume nectar for sugar and carbohydrates (Brødsgaard et al., 2003; Malone and Pham-Delègue, 2001). The amount of pollen consumed by one honey bee larva has previously been estimated to be approximately 1.5 - 2.0 mg (Babendreier et al., 2004), and the amount of pollen consumed by one honey bee adult has previously been estimated to be 3.4 - 4.3 mg (Crailsheim et al., 1992).

Based on the maximum concentration of DvSSJ1 dsRNA in DP23211 maize pollen (2.02×10^{-3} ng/mg) (Table 18), and the maximum amount of pollen consumed by honey bee larvae (2.0 mg) and adults (4.3 mg), the worst-case EEC for DvSSJ1 dsRNA is 4.04×10^{-3} ng/larvae and 8.69×10^{-3} ng/adult bee (Volume 2, Appendix H, Section H1;Table 132). Using the same assumptions, and based on the maximum concentration of the IPD072Aa protein in DP23211 maize pollen (1.3 ng/mg), the worst-case EEC honey bee larvae and adults exposed to the IPD072Aa protein in DP23211 maize pollen is 2.6 ng/larvae and 5.59 ng/bee, respectively (Volume 2, Appendix H, Section H1;Table 134). A refined EEC was not determined for honey bee because exposure using the worst-case assumptions are considered low.

IX-D.2.a.2. Non-target Lepidoptera

The potential exposure of non-target Lepidoptera to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen is assessed due to their importance as beneficial and often charismatic organisms. Based on the concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen, the worst-case exposure of non-target Lepidoptera is considered negligible, as discussed above. Many factors will reduce the actual exposure of non-target Lepidoptera to the DvSSJ1 dsRNA and the IPD072Aa protein from DP23211 maize pollen (e.g., pollen deposition rates and host plant characteristics, protein and dsRNA stability, temporal and spatial overlap, feeding behavior).

Most non-target Lepidoptera larvae do not feed on pollen directly, but indirectly are exposed to pollen as they feed on host plants. For example, Monarch butterfly larvae (*Danaus plexippus*, Lepidoptera: Nymphalidae) utilize milkweed as their host plant (Sears et al., 2001). Therefore, the degree of potential exposure of non-target Lepidoptera to DP23211 maize pollen will depend on the presence of host plants in and adjacent to DP23211 maize fields, as well as the rate of maize pollen deposition. Weed management practices decrease host plant density within the confines of the maize field and field margins (Gathmann et al., 2006) and limit the potential exposure of non-target Lepidoptera to DP23211 maize pollen. Additionally, maize pollen grains typically travel limited distances from the maize field (e.g. Darvas *et al.*, 2004; Pleasants *et al.*,

2001; Sears *et al.*, 2001; US-EPA, 2001a), and environmental conditions, such as heat, relative humidity (Fonseca and Westgate, 2005), and ultra-violet radiation (Koti et al., 2005) may compromise the integrity of the pollen capsule and impact protein and dsRNA stability in pollen. These factors will likely further reduce potential exposure of non-target Lepidoptera to the DvSSJ1 dsRNA and the IPD072Aa protein via ingestion of DP23211 maize pollen.

The degree of spatial overlap of a non-target lepidopteran's foraging range with DP23211 maize fields, as well as the degree of temporal overlap of maize anthesis with sensitive insect life stages, are also important considerations when determining potential exposure to DP23211 maize pollen. The major temporal consideration is the limited time maize pollen shed occurs. The duration of pollen shed can be variable, but typically lasts 1-2 weeks within a single field. In the U.S., pollen shed occurs generally between mid-July and mid-August (Sears et al., 2001). Overlap of time when maize pollen is shed and when potentially sensitive larvae are present may limit exposure. For example, monarchs may be present near maize fields; however, they have multiple generations per year and only a small proportion of the population would be in the larval stage during pollen shed (Oberhauser et al., 2001), which limits the likelihood of population level effects. Based on this timing, the likelihood of the majority of neonates and early instars being exposed to maize pollen is expected to be low. DP23211 maize pollen is therefore not expected to be a major component of their diet.

Based on the maximum concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen, the worst-case EECs for non-target Lepidoptera are 2.02×10^{-3} ng DvSSJ1 dsRNA/mg pollen and 1.3 ng IPD072Aa protein/mg pollen (Volume 2, Appendix H, Section H2;Table 132 and Table 134, respectively). A refined EEC was not determined for non-target Lepidoptera because exposure using the worst-case assumptions is considered negligible.

IX-D.2.a.3. Non-target Coccinellids

The potential exposure of non-target Coccinellidae to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen was assessed due to their importance as beneficial organisms. Furthermore, based on the specificity of the DvSSJ1 dsRNA and the IPD072Aa protein, non-target coccinellids are more likely to be sensitive than NTOs from other orders.

A limited number of coccinellid species may feed directly on maize pollen (pollinivorous species); however, the majority of coccinellids may only incidentally consume pollen when consuming other food. Based on the maximum concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen, the worst-case EECs for non-target coccinellids that consume maize pollen are 2.02×10^{-3} ng DvSSJ1 dsRNA/mg pollen and 1.3 ng IPD072Aa protein/mg pollen. For coccinellids that either directly or indirectly consume DP23211 maize pollen, the worst-case EECs is considered negligible, since they are approximately 18-20X lower than the LC_{50} of the most sensitive target insect, WCR. Several additional factors will further reduce the actual exposure

of non-target coccinellids to DvSSJ1 dsRNA and the IPD072Aa protein below these worst-case EECs.

In addition to the pollen route of exposure, non-target coccinellids may be exposed to DvSSJ1 dsRNA and the IPD072Aa protein from DP23211 maize via a predatory route of exposure. Some coccinellids may also feed directly on the plant tissues (Moser et al., 2008). Predatory or plant-feeding coccinellids would be exposed to a higher concentration of DvSSJ1 dsRNA and the IPD072Aa protein via the predator or plant-feeding route of exposure, relative to pollen exposure, which will be considered the worst-case route of exposure for non-target coccinellids (see VIII.D. Predators and Parasitoids section below; Appendices E and G;Table 132 and Table 134).

IX-D.2.b. Conclusion Regarding Exposure of Pollinators and Pollen Feeders to DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 Maize Pollen

Based on the concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen, the worst-case exposure of non-target pollinators and pollen feeders to the DvSSJ1 dsRNA and IPD072Aa protein is considered negligible, as these exposures are lower than the LC_{50} concentrations of the most sensitive target pest, WCR. Several additional factors will further reduce the actual exposure of non-target pollinators and pollen feeders to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs.

IX-D.3. Soil-dwelling Organisms

The DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize may enter the soil through root exudates, root sloughing, pollen deposition, and post-harvest plant tissue decomposition. Soil-dwelling organisms may be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via ingestion of DP23211 senescent maize tissues (detritivores).

Soil-dwelling decomposers and detritivores are most likely to consume senescent maize tissues that are incorporated into the soil post-harvest (Bachman et al., 2016). The worst-case EEC for soil-dwelling organisms that consume senescent plant material can be calculated based on the maximum concentration of DvSSJ1 dsRNA and the IPD072Aa protein in senescent (R6) whole plant tissue (2.99×10^{-2} ng DvSSJ1 dsRNA/mg and 24 ng IPD072Aa protein/mg (Table 18 and Table 19). A refined EEC for soil-dwelling decomposers and detritivores can be calculated based on the mean concentration of DvSSJ1 dsRNA and the IPD072Aa protein in senescent (R6) whole plant tissue (1.08×10^{-2} ng DvSSJ1 dsRNA/mg and 11 ng IPD072Aa protein/mg (Volume 2, Appendix H, Section H3;Table 18 and Table 19).

Another factor to consider in the exposure assessment of soil-dwelling organisms is the stability of the DvSSJ1 dsRNA and the IPD072Aa protein in soil. Two laboratory studies were conducted to characterize the fate of theDvSSJ1 dsRNA and the IPD072Aa protein in different soil types.

IX-D.3.a. Soil Dissipation of DvSSJ1 dsRNA

The objective of this study was to estimate the dissipation time of DvSSJ1_210 double-stranded RNA (dsRNA) by QuantiGene analysis and the loss of insecticidal activity of DvSSJ1_210 dsRNA in various soils.

DvSSJ1_210 dsRNA was added to three soils: loam, sandy clay loam, and silt loam, which support a range of physicochemical properties and are representative of typical agricultural soils. Dissipation of the DvSSJ1_210 dsRNA in each soil type was assessed using QuantiGene analysis. In addition, the loss of insecticidal activity of DvSSJ1_210 dsRNA was evaluated in six consecutive 14-day bioassays using WCR, by incorporating these soils into artificial diet.

For the QuantiGene analysis, samples from the loam, sandy clay loam, and silt loam soils were extracted after addition of DvSSJ1_210 dsRNA (Day 0) and at multiple intervals over 70 days to assess dissipation of DvSSJ1_210 dsRNA in each soil type over time.

WCR bioassays with each soil type were initiated on the day soils were spiked with DvSSJ1_210 dsRNA (Day 0) and at 7-day intervals for the first three bioassays and 14 day intervals for the remaining bioassays. For each bioassay, diets were prepared by collection of one randomly selected aliquot of the DvSSJ1_210 dsRNA-spiked soil samples and one randomly selected aliquot of the control soil samples for each soil type. Aliquots were then incorporated into artificial diet. WCR larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Loam soil control spiked with RNase-free water
- Treatment 2: Sandy clay loam soil control spiked with RNase-free water
- Treatment 3: Silt loam soil control spiked with RNase-free water
- Treatment 4: Loam soil spiked with DvSSJ1_210 dsRNA
- Treatment 5: Sandy clay loam soil spiked with DvSSJ1_210 dsRNA
- Treatment 6: Silt loam soil spiked with DvSSJ1_210 dsRNA

For each bioassay, each diet was provided to 30 individual WCR. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed every three to four days after each bioassay was initiated with additional soil samples, aged an additional three or four days, collected and prepared as described above. After a total of 14 days, each bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality results were used to demonstrate the loss of activity for the DvSSJ1_210 dsRNA in each soil type.

Dissipation of the DvSSJ1_210 dsRNA was observed via QuantiGene analysis with approximately 80% reduction within 24 hours in all three soil types (summarized in Table 102). Bioassays with WCR showed loss of bioactivity within 30 days in sandy clay loam and silt loam (Table 100, Table

101) soils. Bioactivity in the loam soil showed a reduction in potency from day 0 to day 55 (Table 99). Based on the demonstrated dissipation of the DvSSJ1_210 dsRNA via QuantiGene analysis and the loss of insecticidal activity in a variety of soil types, the DvSSJ1 dsRNA is unlikely to persist or accumulate in soil.

Acceptability criteria, individual assay results, and materials and methods are presented in Volulme 2, Appendix J.

Table 99. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Loam Soil

Timepoint (hours)	0	4	24	75	168	341	576	760	1013	1346	1683
Percent of Time Zero	100%	31%	9%	4%	3%	3%	2%	2%	3%	3%	3%

Table 100. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Sandy Clay Loam Soil

Timepoint (hours)	0	4	24	75	168	341	576	760	1013
Percent of Time Zero	100%	56%	22%	8%	4%	2%	1%	1%	1%

Table 101. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Silt Loam Soil

Timepoint (hours)	0	4	24	75	168	341	576	760	1013
Percent of Time Zero	100%	40%	18%	13%	9%	7%	7%	6%	6%

Table 102. Summary of DvSSJ1 dsRNA WCR Soil Dissipation Mortality Results (Initiated with Day 0 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Control Loam Soil	28[a]	7.14	26	0.681 ± 0.397	0.2 - 1.8
2	Control Sandy Clay Loam Soil	30	3.33	29	0.738 ± 0.506	0.2 - 2.4
3	Control Silt Loam Soil	30	0.00	30	0.980 ± 0.639	0.1 - 2.4
4	Loam Soil Spiked with DvSSJ1_210 dsRNA	30	100	0	NA	NA
5	Sandy Clay Loam Soil Spiked with DvSSJ1_210 dsRNA	29[a]	79.3	6	0.300 ± 0.245	0.1 - 0.7
6	Silt Loam Soil Spiked with DvSSJ1_210 dsRNA	26[a]	73.1	7	0.357 ± 0.162	0.1 - 0.5

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% soil incorporated into dry carrier (by carrier dry weight). Treatments 1, 2, and 3 contained soil spiked with RNase-free water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

IX-D.3.b. Soil Dissipation of IPD072Aa Protein

The objective of the soil dissipation study was to estimate the dissipation time of IPD072Aa protein in various soils by western blot analysis and the loss of insecticidal activity of IPD072Aa protein.

IPD072Aa protein was added to three soils: loam, sandy clay loam, and silt loam, which support a range of physicochemical properties and are representative of typical agricultural soils.

Dissipation of the IPD072Aa protein in each soil type was visually confirmed via western blot analysis. In addition, the loss of insecticidal activity of IPD072Aa protein was evaluated in two consecutive 7-day bioassays using WCR, by incorporating these soils into artificial diet.

For the western blot analysis, samples from the loam, sandy clay loam, and silt loam soils were extracted on days 0, 1, 2, 3, 4, 7, and 14 after addition of IPD072Aa protein. Disappearance of IPD072Aa protein was visually assessed to confirm dissipation of IPD072Aa protein in each soil type over time.

WCR bioassays with each soil type were initiated on the day soils were spiked with IPD072Aa protein (Day 0) and after a period of 7 days. For each bioassay, diets were prepared by collection of three randomly selected aliquots of the IPD072Aa protein-spiked soil samples and three control soil samples for each soil type. Aliquots were then pooled by soil type and treatment, and incorporated into artificial diet. WCR larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Loam soil spiked with IPD072Aa protein
- Treatment 2: Loam soil control (spiked with ultrapure water)
- Treatment 3: Sandy clay loam soil spiked with IPD072Aa protein
- Treatment 4: Sandy clay loam soil control (spiked with ultrapure water)
- Treatment 5: Silt loam soil spiked with IPD072Aa protein
- Treatment 6: Silt loam soil control (spiked with ultrapure water)

Each diet was provided to 30 individual WCR. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed four days after each bioassay was initiated with additional soil samples, aged an additional four days, collected and prepared as described above. After a total of 7 days, each bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality results were used to demonstrate the loss of activity for the IPD072Aa protein in each soil type.

Collectively, visual confirmation using western blot analyses and mortality results from WCR bioassays both support rapid dissipation of the IPD072Aa protein within 7 days across multiple soil types (Table 103 and Table 104). The dissipation of IPD072Aa protein in these diverse soil types occurred in less than 7 days, and the protein is therefore unlikely to persist or accumulate in the field where DP23211 maize is cultivated.

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix K.

Table 103. Summary of IPD072Aa Protein WCR Soil Dissipation Mortality Results (Initiated with Day 0 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Loam Soil Spiked with IPD072Aa Protein	28[a]	67.9	9	0.0889 ± 0.0601	0.0 - 0.2
2	Control Loam Soil	30	0	30	0.350 ± 0.117	0.1 - 0.6
3	Sandy Clay Loam Soil Spiked with IPD072Aa Protein	29[a]	58.6	12	0.158 ± 0.0900	0.1 - 0.4
4	Control Sandy Clay Loam Soil	28[a]	3.57	27	0.389 ± 0.167	0.1 - 0.9
5	Silt Loam Soil Spiked with IPD072Aa Protein	28[a]	50.0	14	0.143 ± 0.0852	0.1 - 0.4
6	Control Silt Loam Soil	29[a]	0	29	0.448 ± 0.124	0.2 - 0.7

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% pooled soil incorporated into dry carrier (by carrier dry weight). Treatments 2, 4, and 6 contained soil spiked with ultrapure water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 104. Summary of IPD072Aa Protein WCR Soil Dissipation Mortality Results (Initiated with Day 7 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Loam Soil Spiked with IPD072Aa Protein	30	0	30	0.223 ± 0.125	0.1 - 0.5
2	Control Loam Soil	30	0	30	0.333 ± 0.132	0.1 - 0.6
3	Sandy Clay Loam Soil Spiked with IPD072Aa Protein	29[a]	0	29	0.352 ± 0.143	0.1 - 0.7
4	Control Sandy Clay Loam Soil	30	0	30	0.343 ± 0.125	0.1 - 0.6
5	Silt Loam Soil Spiked with IPD072Aa Protein	30	6.67	28	0.325 ± 0.140	0.1 - 0.7
6	Control Silt Loam Soil	30	0	30	0.373 ± 0.184	0.1 - 0.7

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% pooled soil incorporated into dry carrier (by carrier dry weight). Treatments 2, 4, and 6 contained soil spiked with ultrapure water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

IX-C.3.b. Conclusion Regarding Exposure of Soil Dwelling Organisms to DvSSJ1 dsRNA and IPD072Aa

Soil-dwelling decomposers and detritivores are most likely to consume senescent maize tissues that are incorporated into the soil post-harvest (Bachman et al., 2016). The worst-case EEC for soil-dwelling organisms that consume senescent plant material can be calculated based on the maximum concentration of DvSSJ1 dsRNA and the IPD072Aa protein in senescent (R6) whole plant tissue (2.99×10^{-2} ng DvSSJ1 dsRNA/mg and 24 ng IPD072Aa protein/mg (Table 18 and Table 19). A refined EEC for soil-dwelling decomposers and detritivores can be calculated based on the mean concentration of DvSSJ1 dsRNA and the IPD072Aa protein in senescent (R6) whole plant tissue (1.08×10^{-2} ng DvSSJ1 dsRNA/mg and 11 ng IPD072Aa protein/mg (Volume 2, Appendix H, Section H3;Table 18 and Table 19).

Dissipation of the DvSSJ1_210 dsRNA was observed via QuantiGene analysis with approximately 80% reduction within 24 hours in all three soil types and bioassays with WCR showed loss of bioactivity within 30 days in sandy clay loam and silt loam soils. Bioactivity in the loam soil showed a reduction in potency from day 0 to day 55. Based on the demonstrated dissipation of the DvSSJ1_210 dsRNA via QuantiGene analysis and the loss of insecticidal activity in a variety of soil types, the DvSSJ1 dsRNA is unlikely to persist or accumulate in soil.

Visual confirmation using western blot analyses and mortality results from WCR bioassays both support rapid dissipation of the IPD072Aa protein within 7 days across multiple soil types. The dissipation of IPD072Aa protein in these diverse soil types occurred in less than 7 days, and the protein is therefore unlikely to persist or accumulate in the field where DP23211 maize is cultivated.

IX-D.4. Aquatic Organisms

Potential exposure of non-target aquatic organisms to PIPs in GM crops has been considered previously, with movement of senescent tissue identified as the most likely route of exposure for aquatic organisms (Carstens et al., 2010). Although aquatic habitats may be located near agricultural areas, exposure of aquatic organisms to biotech crops is limited temporally and spatially (Bachman et al., 2016) and aquatic exposure to *Bt* corn is extremely small (US-EPA, 2010a). The specificity and environmental fate of the DvSSJ1 dsRNA and the IPD072Aa protein, as well as the worst-case assumptions about potential input of maize tissue in aquatic environments, can be used to help inform the risk assessment for aquatic organisms (Carstens et al., 2012).

The DvSSJ1 dsRNA has been shown to be highly specific, with activity limited to species within the genus *Diabrotica* and family Chrysomelidae (Table 67)(Hu and Anderson, 2019)(Hu and Anderson, 2019). The IPD072Aa protein has been shown to be specific, with activity limited to within the order Coleoptera (Boeckman *et al.*, 2019).

For dsRNA, previously published studies on the environmental fate of dsRNA have provided evidence that dsRNAs do not persist in soil (Dubelman et al., 2014; Fischer et al., 2016) or water (Albright III et al., 2017; Fischer et al., 2017). Based on dissipation of the DvSSJ1 dsRNA in different soil types (Volume 2, Appendix J; Table 99, Table 100, Table 101, and Table 102), this dsRNA is unlikely to persist or accumulate in the environment. Similarly, the IPD072Aa protein dissipates in different soil types (Volume 2, Appendix K; Table 103 and Table 104) and is unlikely to persist or accumulate in the environment.

The worst-case EEC for aquatic organisms to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize tissues was estimated using the EPA standard agricultural field-farm pond model (also called the US EPA standard pond model (Jones et al., 2004)). The EPA standard agricultural field-farm pond model provides estimates for predicting pesticide runoff concentrations and uses the assumptions that runoff from a 10-hectare (ha) field is deposited in a 1-ha pond (2 meters deep; equivalent to 20,000,000 L of water). This approach has previously been adapted to model a conservative exposure scenario for aquatic NTOs to newly expressed proteins in GM crops (Carstens et al., 2012; Raybould and Vlachos, 2011; Wolt and Peterson, 2010) and is also suitable for assessing dsRNA in GM crops (US-EPA, 2017).

The worst-case EECs for aquatic organisms can be calculated based on the highest mean whole plant tissue concentration (highest mean value from any growth stage) of DvSSJ1 dsRNA and the IPD072Aa protein (2.19×10^{-2} ng DvSSJ1 dsRNA/mg in R1 whole plant; 11 ng IPD072Aa protein/mg in R6 whole plant). Based on the assumptions of the EPA farm pond model, the worst-case EECs for aquatic organisms are 2.46×10^{-4} mg/l and 0.124 mg/l for the DvSSJ1 dsRNA and the IPD072Aa protein (Table 132 and Table 134, respectively). Although these worst-case assumptions are extremely conservative, the EPA has previously noted that more refined exposure estimates are generally not needed unless this screening level calculation indicates exposure levels above a level of concern (US-EPA, 2017). Therefore, a refined EEC was not determined for aquatic organisms because the worst-case EECs are considered low. Several additional factors will further reduce the actual exposure of aquatic organisms to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs.

X-D.5. Predators and Parasitoids

A predator or parasitoid may be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via consumption of prey that has previously consumed tissue from a DP23211 maize plant. Because a predator does not feed directly on the maize plant, one factor to consider in the exposure assessment for predators or parasitoids is the amount of DvSSJ1 dsRNA and IPD072Aa protein that transfers and accumulates in the prey. Secondary exposures via prey are influenced not only by the rates of ingestion, digestion and excretion of plant material by the prey (see, Rose, 2007 for review), but also by the stability of DvSSJ1 dsRNA and the IPD072Aa protein within the prey. Some predators (for example coccinellids) may also feed directly on the plant tissues (Moser et al., 2008).

The worst-case EEC for predators and parasitoids assumes: 1) that 100% of the dsRNA or protein in the GM plant transfers to the prey and then subsequently is transferred to the predator (no degradation or loss); and 2) that predators are exposed to the maximum concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (maximum from any above-ground plant tissue and from any growth stage). This route of exposure also is used for coccinellid species that may feed directly on the plant tissues, since the EEC is calculated based on 100% plant tissue concentration. Based on these assumptions, the worst-case EEC is 0.113 ng/mg and 39 ng/mg for the DvSSJ1 dsRNA and the IPD072Aa protein (Table 132 and Table 134, respectively).

The refined EEC assumes: 1) that 100% of the dsRNA or protein in the GM plant transfers to the prey then subsequently is transferred to the predator (no degradation or loss); and 2) that predators are exposed to the mean concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (highest mean value from any above-ground plant tissue and from any growth stage). Based on these assumptions, the refined EEC is 0.0646 ng/mg and 16 ng/mg for the DvSSJ1 dsRNA and the IPD072Aa protein (Table 132 and Table 134, respectively). Several factors will

reduce the actual exposure of predators and parasitoids to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs, therefore the refined EEC represents a more environmentally realistic exposure for predators and parasitoids.

X-D.6. Insectivorous Birds

Some wild birds are insectivorous and could be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via prey (tri-tropic transfer). The factors that may limit the potential exposure of wild birds to the DvSSJ1 dsRNA and the IPD072Aa protein via prey are discussed above for predators and parasitoids.

For insectivorous birds, the worst-case EEC assumes: 1) that 100% of the DvSSJ1 dsRNA or IPD072Aa protein in the GM plant transfers to the prey and then subsequently to the wild bird (no degradation or loss); and 2) that predators are exposed to the maximum concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (maximum from any above-ground plant tissue and from any growth stage). Based on these assumptions, the worst-case EEC is 0.113 ng/mg and 39 ng/mg for the DvSSJ1 dsRNA and the IPD072Aa protein (Table 132 and Table 134 respectively).

The refined EEC assumes: 1) that 100% of the DvSSJ1 dsRNA or IPD072Aa protein in the GM plant transfers to the prey and then subsequently to the predator (no degradation or loss); and 2) that predators are exposed to the mean concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (highest mean value from any above-ground plant tissue and from any growth stage). Based on these assumptions, the refined EEC is 0.0646 ng/mg and 16 ng/mg for the DvSSJ1 dsRNA and the IPD072Aa protein (Table 132 and Table 134, respectively). Several factors will reduce the actual exposure of insectivorous birds to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs, therefore the refined EEC represents a more environmentally realistic exposure for predators and parasitoids.

X-D.7. Granivorous Mammals

Granivorous wildlife (e.g., rodents) may be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein by feeding on DP23211 maize grain. Several factors may limit the potential exposure of granivorous mammals to the DvSSJ1 dsRNA and the IPD072Aa from DP23211 maize. Wild rodents are unlikely to consume only maize grain, as they typically feed on a variety of cereal seeds. Also, the concentration of the DvSSJ1 dsRNA and the IPD072Aa in DP23211 maize grain is low (maximum concentrations in grain are 1.09×10^{-2} and 4.8 ng/mg, respectively (Table 18 and Table 19). Furthermore, there are barriers to uptake of dsRNA that likely limit potential exposure of mammals to the DvSSJ1 dsRNA (see Section VII-A.3.b. Mammalian Barriers to Exposure to DvSSJ1 dsRNA Consumed in Food and Feed).

For wild rodents that consume grain, a worst-case EEC assumes consumption of a diet that contains 73% maize grain (Raybould et al., 2007), and is based on the maximum concentration of the DvSSJ1 dsRNA and the IPD072Aa protein in maize grain. A daily dietary dose (DDD) for wild rodents can be calculated (Raybould and Vlachos, 2011), which accounts for food intake, body weight, and DvSSJ1 dsRNA and the IPD072Aa protein concentration in grain (see Appendix H). The worst-case EEC for wild mammals exposed to DvSSJ1 dsRNA in DP23211 maize is 2.6×10^{-3} mg/kg body weight (Table 132). The worst-case EEC for wild mammals exposed to the IPD072Aa protein in DP23211 maize is 1.156 mg/kg body weight (Table 134). A refined EEC was not determined for wild mammals because exposure using the worst-case assumptions are considered low. Several additional factors will further reduce the actual exposure of granivorous mammals to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs.

XI. Non-Target Organism Hazard Assessment

Hazard assessments should be conducted using a tiered testing system (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2008; US-EPA, 2010b). Early tier tests are laboratory-based bioassays designed to simulate worst-case scenarios, targeting 10X the EEC (Rose, 2007; US-EPA, 2010b). If no significant hazard (i.e., greater than 50% mortality) is observed at high laboratory exposures, then the risk of adverse effects at environmentally relevant concentrations is likely to be low (Rose, 2007). Only when the early tier hazard study detects an effect should the next tier study be considered (US-EPA, 2010a), taking into account the level of concern associated with the environmental hazard, potential mitigation options to alleviate the concern, and the ability to conduct more complex higher tier studies (e.g., Romeis et al., 2008; Rose, 2007).

Problem formulation and the exposure assessments were used to guide hazard testing. Early tier laboratory studies were conducted using representative surrogate species at concentrations exceeding the worst-case EECs to assess for potential adverse impacts of the DvSSJ1 dsRNA and the IPD072Aa protein. Selection criteria for relevant surrogate species included phylogenetic relation to the target insects, ecological function, presence in the agroecosystem, and practical considerations such as availability of the test species, amenability to testing, and availability of standard test methods (Carstens et al., 2012; Carstens et al., 2014; Romeis et al., 2011; Romeis et al., 2013). Organisms selected for these studies were comprised of species representative of pollinators and pollen feeders, soil-dwelling organisms, predators and parasitoids, granivorous mammals and insectivorous birds.

The early-tier NTO studies conducted for the DvSSJ1 dsRNA and the IPD072Aa protein are summarized and the median lethal concentration (LC_{50}) or lethal dose (LD_{50}), no-observed-effect-concentration (NOEC), no-observed-effect-dose (NOED), or no-observed-effect-dietary-dose (NOEDD) are reported below. The margin of exposure (MOE) for DvSSJ1 dsRNA and the IPD072Aa protein using worst-case or refined EECs for DP23211 maize tissues are also included in the summaries below. Further details of the calculation of EECs and MOEs for DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize are provided in Appendix H.

Dry weight concentrations were used for the risk assessment of DvSSJ1 dsRNA and IPD072Aa protein in DP23211 maize, and this method is the most appropriate method to consider as part of the risk assessment. Using DvSSJ1 dsRNA and IPD072Aa protein concentrations based on fresh weight adds variability to the concentrations caused by varying levels of maize tissue moisture. Lyophilizing tissue samples prior to protein extraction and analysis provides a robust and conservative measurement of protein concentration in various tissues. This approach has a long history of use in GE crop characterization. No conversion factors were used to derive dry weight concentrations for the IPD072Aa protein.

Given that these measurements were collected for each tissue type at the time of analysis, the DvSSJ1 dsRNA concentrations are accurate for both the quantified fresh weight concentration and the calculated dry weight concentration. As the IPD072Aa protein was extracted from lyophilized DP23211 maize tissue, it is most appropriate to only report dry weight expression values for the IPD072Aa protein in the relevant tissues used for the risk assessment of DP23211 maize. Attempting to retroactively apply a dry weight: fresh weight conversion factor for the IPD072Aa protein is not warranted, would likely introduce more variation in to the data set.

From an environmental safety perspective, all environmental exposure assumptions used dry weight concentrations of plant tissue and fresh weight concentrations for the bioassay diets. This approach provides a conservative margin of exposure, which builds additional conservancy into the risk assessment overall.

XI-A. Pollinators and Pollen Feeders

Pollinators and pollen feeders can be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen by directly feeding on maize pollen or by incidentally feeding on maize pollen (i.e., pollen deposited on host plant leaves). Early tier laboratory studies were conducted to assess hazard to the pollinator and pollen feeder functional group.

XI-A.1. Honey bee

Studies were performed to evaluate the dietary effect of DvSSJ1 dsRNA or the IPD072Aa protein on honey bee (*Apis mellifera*) larvae and adults.

Honey bee larvae were used in a 22 day bioassay (following OECD Guidance Document No. 239) with a targeted concentration of 0.0040 µg DvSSJ1 dsRNA/larva (Table 132)(Patnaude, 2019; Table 2)(Patnaude, 2019; Table 2). No effects on larval survival, pupal survival, adult emergence, or adult weight at emergence were observed. The NOED for honey bee larvae is 0.0040 µg DvSSJ1 dsRNA/larval cell (equivalent to 4.0 ng DvSSJ1 dsRNA/larvae cell) (Table 105). The MOE, based on a NOED of 4.0 ng DvSSJ1 dsRNA/larvae, is 990X the worst-case EEC for honey bee larvae exposed to the DvSSJ1 dsRNA in DP23211 maize pollen (Table 132).

Methods and materials for the bioassay evaluation of Honey bee are presented in Volume 2, Appendix E, Section E2.

Table 105. Honey Bee Larval Toxicity Test, Repeated Exposure to DvSSJ1_210 Double-Stranded RNA - Endpoint Summary

Endpoint	Based on Nominal Cumulative Dose (ng a.i./larva)	
	NOED[a]	LOED[b]
3 - 8-Day Larval Survival	4	>4
8 - 22-Day Pupal Survival	4	>4
3 - 22-Day Adult Emergence	4	>4
Adult Weight at Emergence	4	>4

[a] NOED = No-Observed-Effect Dose

[b] LOED = Lowest-Observed-Effect Dose

Honey bee adults (≤ 2-day old emerged) were exposed (following OECD 245) to a mean daily dose of 0.026 µg DvSSJ1 dsRNA, per bee per day, for 14 days (Table 132). No effects on adult body weight or survival were observed. The NOEDD for honey bee adults is 0.026 µg DvSSJ1 dsRNA/bee/day (equivalent to 26 ng DvSSJ1 dsRNA/bee/day) (Table 106). The MOE, based on a NOEDD of 26 ng DvSSJ1 dsRNA/bee is 2,993X the worst-case EEC for honey bee adults exposed to DvSSJ1 RNA in DP23211 maize (Table 132).

Table 106. 14-Day Oral Exposure of Honey Bees to DvSSJ1_210 double-stranded RNA - Endpoint Summary

Endpoint	Nominal Diet (mg a.i./kg)	Calculated Mean Daily Dose (ng a.i./bee/day)
	10-day Percent Survival	
NOEC/NOEDD	0.81	26
LOEC/LOEDD	>0.81	>26
	14-day Percent Survival	
NOEC/NOEDD	0.81	26
LOEC/LOEDD	>0.81	>26
	14-day Live Adult Weight	
NOEC/NOEDD	0.81	26
LOEC/LOEDD	>0.81	>26

Honey bee larvae were used in a 22-day bioassay (following OECD Guidance Document No. 239) with targeted concentrations of 0.10 and 0.20 µg IPD072Aa protein/larva (Table 133). No effects on larval survival, pupal survival, adult emergence, or adult weight at emergence were observed. The NOED for honey bee larvae is 0.20 µg IPD072Aa protein/larval cell (equivalent to 200 ng IPD072Aa protein/larvae cell) (Table 107). The MOE, based on a NOED of 200 ng IPD072Aa protein/larvae, is 77X the worst-case EEC for honey bee larvae exposed to the IPD072Aa protein in DP23211 maize pollen (Table 134).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix G, Section G2.

Table 107. Honey Bee Larval Toxicity Test, Repeated Exposure to IPD072Aa Protein - Endpoint Summary

Endpoint	Based on Nominal Cumulative Dose (ng a.i./larva)	
	NOED[a]	LOED[b]
3 - 8-Day Larval Survival	200	>200
8 - 22-Day Pupal Survival	200	>200
3 - 22-Day Adult Emergence	200	>200
Adult Weight at Emergence	200	>200

[a] NOED = No-Observed-Effect Dose
[b] LOED = Lowest-Observed-Effect Dose

Honey bee adults (≤ 2-day old emerged) were exposed (following OECD 245) to a mean daily dose of 1.3 µg IPD072Aa protein, per bee per day, for 10 days (Table 133). No effects on adult body weight or survival were observed. The NOEDD for honey bee adults is 1.3 µg IPD072Aa protein/bee/day (equivalent to 1,300 ng IPD072Aa protein/bee/day) (Table 108). The MOE, based on a NOEDD of 1,300 ng IPD072Aa protein/bee, is 233X the worst-case EEC for honey bee adults exposed to the IPD072Aa protein in DP23211 maize pollen (Table 134).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix G, Section G3.

Table 108. 10-Day Oral Exposure of Honey Bees to IPD072Aa Protein - Endpoint Summary

Endpoint	Nominal Diet (mg a.i./kg)	Calculated Mean Daily Dose (µg a.i./bee/day)
	Percent Survival	
NOEC/NOEDD	41	1300
LOEC/LOEDD	>41	>1300
	Live Adult Weight	
NOEC/NOEDD	41	1300
LOEC/LOEDD	>41	>1300

XI-A.1.a. Honey Bee - Non Target Hazard Assessment Conclusions:

No hazard was detected in early-tier hazard assessments on honey bee larvae after exposure to 990X or 77X the worst-case EEC of the DvSSJ1 dsRNA the IPD072Aa protein, respectively. No hazard was detected honey bee adults after exposure to 2,993X or 233X the worst-case EEC of

the DvSSJ1 dsRNA the IPD072Aa protein, respectively. The overall MOE values indicate that DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize are unlikely to be harmful to honey bees at environmentally realistic concentrations.

XI-A.2. Non-target Lepidoptera

Based on the concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen, the worst-case exposure of non-target Lepidoptera to the DvSSJ1 dsRNA is considered negligible (2.02×10^{-3} ng DvSSJ1 dsRNA/mg pollen or 1.3 ng IPD072Aa protein/mg pollen (Table 132 and Table 134). Several additional factors will further reduce the actual exposure of non-target Lepidoptera to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs.

As part of characterization of the spectrum of activity of DvSSJ1 dsRNA and the IPD072Aa protein, four Lepidoptera species, ECB, CDM, PL, and CEW, were assessed to test for cross-order activity (Table 132 and Table 133). No adverse effects on survival was observed at 1 ng DvSSJ1 dsRNA/mg diet and 1000 ng IPD072Aa protein/mg diet, which are concentrations that approximate 495X and 769X the worst-case EECs for non-target Lepidoptera (Table 132 and Table 134).

Furthermore, the most sensitive target species tested is a useful indicator of potential effects on NTOs (US-EPA, 2001a). The worst-case EEC for non-target Lepidoptera was also compared to the LC_{50} of the most sensitive target pest tested, WCR. The LC_{50} of DvSSJ1 dsRNA for WCR is 0.036 ng/mg (Table 39), which is 18X higher than the worst-case EEC for non-target Lepidoptera (Table 132). The LC_{50} of the IPD072Aa protein for WCR is 26 ng/mg (Table 68), which is 20X higher than the worst-case EEC for non-target Lepidoptera (Table 134).

XI-A.2.a. Non-Target Lepidoptera – Non-Target Hazard Assessment Conclusions:

Considering the lack of activity of the DvSSJ1 dsRNA and the IPD072Aa protein observed on Lepidoptera as part of spectrum of activity testing, as well as negligible potential for exposure, the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize is unlikely to be harmful to non-target Lepidoptera at environmentally realistic concentrations.

XI-B. Soil-Dwelling Organisms

Studies were performed to evaluate the dietary effect of DvSSJ1 dsRNA and the IPD072Aa protein on Springtail, which was selected to represent a non-target detritivore.

Springtail adults were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 28 days (Table 131). No adverse effect on springtail survival or reproduction was observed (Table 109 and Table 110). The MOEs, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 33X the worst-case EEC and 93 X the refined EEC (Table 132).

Methods and materials for the bioassay evaluation of Springtail are presented in Volume 2, Appendix E, Section E1.

Table 109. Summary Analysis of Springtai/ DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	80	3	3.75	--
2	Test Diet	1	81[a]	0	0	1.0000
3	Positive Control Diet	0[b]	80	77	96.3	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.
[a] One jar for Treatment 2 contained 11 organisms.
[b] Treatment 3 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

Table 110. Summary Analysis of Springtail DvSSJ1 dsRNA Bioassay Reproduction Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Mean Number of Offspring (95% Confidence Interval)	Range (mg)	CV	P-Value
1	Bioassay Control Diet	0	319 (283-360)	268 - 399	14.5	--
2	Test Diet	1	345 (306-389)	264 - 462	19.6	0.8290
3	Positive Control Diet	0[a]	0	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no offspring recorded for *Folsomia candida* in Treatment 3.
[a] Treatment 3 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

Springtail adults were exposed to diet containing a target concentration of 500 ng IPD072Aa protein/mg diet for 28 days (Table 133). While a statistically significant difference was observed in mean reproduction, this result was not considered to be biologically relevant, based on acceptability guidelines for collembolan reproduction established by OECD, as well as the overlapping range of offspring. No effects on survival were observed (Table 111 and Table 112). The MOE, based on a NOEC of 500 ng IPD072Aa protein/mg diet, is 21X the worst-case EEC and 45 X the refined EEC (Table 134).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix G, Section G1.

Table 111. Summary Analysis of Springtail IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	81[a]	0	0.00	--
2	Test Diet	500	80	2	2.50	0.2453
3	Heat-treated Control Diet	500	80	1	1.25	--
4	Positive Control Diet	0[b]	80	74	92.5	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet dry weight.
[a] One jar for Treatment 1 contained 11 organisms.
[b] Treatment 4 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

Table 112. Summary Analysis of Springtail IPD072Aa Protein Bioassay Reproduction Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Mean Number of Offspring Per Jar (95% Confidence Interval)	Range	%CV	P-Value
1	Bioassay Control Diet	0	460 (409 - 517)	356 - 571	15.7	--
2	Test Diet	500	394 (350 - 444)	317 - 554	18.5	0.0344[a]
3	Heat-treated Control Diet	500	420 ± 94.8[b]	269 - 599	--	--
4	Positive Control Diet	0[c]	0	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet dry weight. Not applicable (NA); there were no offspring recorded for *Folsomia candida* in Treatment 4.
[a] A statistically significant difference (P-value < 0.05) was observed.
[b] Standard deviation is provided for mean values not subjected to linear mixed model analysis.
[c] Treatment 4 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

XI-B.1. Soil-Dwelling Organisms – Non-Target Hazard Assessment Conclusions:

No hazard was detected in the bioassay hazard assessment of a representative non-target detritivore after exposure to 33X and 21X the worst-case EEC of the DvSSJ1 dsRNA and IPD072Aa protein, respectively (Table 132 and Table 134). The refined EECs represent more realistic environmental exposures, and based on the overall MOE values, the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize are unlikely to be harmful to non-target soil dwelling organisms at environmentally realistic concentrations.

XI-.C. Aquatic Non-Target Organisms

Based on the assumptions of the EPA farm pond model, the worst-case EECs for aquatic organisms are 2.46×10^{-4} mg/l and 0.124 mg/l for the DvSSJ1 dsRNA and the IPD072Aa protein, respectively.

The worst-case EECs for non-target aquatic organisms were compared to the LC_{50} of the most sensitive target pest tested, WCR. The most sensitive target species tested is a useful indicator of

potential effects on NTOs (US-EPA, 2001a). The LC_{50} of DvSSJ1 dsRNA for WCR is 0.036 ng/mg (Table 39), which is 146X higher than the worst-case EEC for aquatic organisms (Table 132) The LC_{50} of the IPD072Aa protein for WCR is 26 ng/mg (Table 68), which is 210X higher than the worst-case EEC for aquatic organisms (Table 134).

XI-C.1. Aquatic Non-Target Organisms – Non-Target Hazard Assessment Conclusions:

Tier I hazard studies on aquatic species for the DvSSJ1 dsRNA and the IPD072Aa protein were not conducted based on negligible potential for exposure. The DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize is unlikely to be harmful to non-target aquatic organisms at environmentally realistic concentrations.

XI-D. Predators and Parasitoids

Predators and parasitoids are an important group of NTOs that may be found within the agroecosystem. Early tier laboratory studies were conducted to assess the dietary effect of DvSSJ1 dsRNA and the IPD072Aa protein to this functional group using suitable surrogate species.

XI-D.1. Green Lacewing (*Chrysoperla rufilabris)*

Green Lacewing larvae were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 21 days (Table 131). No adverse effects on survival or pupation of green lacewing were observed (Table 113 and Table 114). The MOE, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 9X the worst-case EEC and 15X the refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey (Table 132).

Methods and materials for the bioassay evaluation of Green Lacewing are presented in Appendix E, Section E5.

Table 113. Summary Analysis of Green Lacewing DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	39[a]	1	2.56	--
2	Test Diet	1	40	1	2.50	0.7595
3	Positive Control Diet	0[b]	40	40	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight

Table 114. Summary Analysis of Green Lacewing DvSSJ1 dsRNA Bioassay Pupation Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Alive Larvae	Number of Pupated Larvae	Pupation (%)
1	Bioassay Control Diet	0	38	38	100
2	Test Diet	1	39	39	100
3	Positive Control Diet	0 [a]	0	-	-

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a]Treatment 3 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight.

Green Lacewing larvae were exposed to diet containing a target concentration of 500 ng IPD072Aa protein/mg diet for 21 days (Table 133). No adverse effects on survival or pupation of green lacewing were observed (Table 115 and Table 116). The MOE, based on a NOEC of 500 ng IPD072Aa protein/mg diet, is 13X the worst-case EEC and 31X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey (Table 134).

Acceptability criteria, individual assay results, and materials and methods are presented in Appendix G, Section G5.

Table 115. Summary Analysis of Green Lacewing IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	40	5	12.5	--
2	Test Diet	500	39[a]	4	10.3	0.7465
3	Heat-treated Control Diet	500	40	2	5.00	--
4	Positive Control Diet	0[b]	35[c]	35	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet wet weight.
[a] Organisms lost in transfer were not included in the total number of observations for a given treatment.
[b] Treatment 4 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight.
[c] Treatment 4 was infested with 35 organisms due to lower than expected insect hatching.

Table 116. Summary of Green Lacewing IPD072Aa Protein Bioassay Pupation Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Alive Larvae	Number of Pupated Larvae	Pupation (%)
1	Bioassay Control Diet	0	35	35	100
2	Test Diet	500	35	35	100
3	Heat-treated Control Diet	500	38	38	100
4	Positive Control Diet	0[a]	0	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet wet weight.
[a] Treatment 4 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight.

XI-D.2. Pink spotted lady Beetle (CMAC)

CMAC neonates were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 28 days (Table 131). No adverse effects on survival, weight, or number of days to adult emergence of CMAC were observed (Table 117, Table 118, and Table 119). The MOE, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 9X the worst-case EEC and 15X the refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey (Table 132).

Acceptability criteria, individual assay results, and materials and methods are presented in Volume 2, Appendix D, Section D11.

Table 117. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	1	29[a]	3	10.3	0.1124
3	Positive Control Diet	0[b]	28[a]	28	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

Table 118. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	12.6 (11.7 - 13.5)	7.8 - 18.0	--
2	Test Diet	1	26	13.0 (12.3 - 13.7)	9.2 - 16.8	0.7611
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CMAC in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

Table 119. Summary Analysis of CMAC DvSSJ1 dsRNA BioassayDays to Adult Emergence Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	30	15.0 ± 1.17	15	14 - 20	--	--
2	Test Diet	1	26	14.6 ± 0.983	14	13 - 17	0.9429	0.2679
3	Positive Control Diet	0[a]	0	NA	NA	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CMAC in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

CMAC neonates were exposed to diet containing a target concentration of 100, 500, and 1000 ng IPD072Aa protein/mg diet for 28 days (Table 133). No adverse effects on survival, weight, or number of days to adult emergence of CMAC were observed at 100 ng IPD072Aa protein/mg diet (Table 120, Table 121, and Table 122). The MOE, based on a NOEC of 100 ng IPD072Aa protein/mg diet, is 3X the worst-case EEC and 6X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein/mg in DP23211 maize via prey (Table 134).

Table 120. Summary Analysis of CMAC IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	29[a]	2	6.90	--
2	Test Diet	100	30	2	6.67	0.7070
3	Test Diet	500	30	8	26.7	0.0449[b]
4	Test Diet	1000	30	11	36.7	0.0061[b]
5	Heat-treated Control Diet	1000	29[a]	3	10.3	--
6	Positive Control Diet	0	30	30	100	--.

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight. Treatment 6 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

Table 121. Summary Analysis of CMAC IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	27	12.0 (11.3 - 12.7)	5.4 - 14.9	--
2	Test Diet	100	28	12.1 (11.4 - 12.8)	9.0 - 15.7	0.5424
3	Test Diet	500	22	10.7 (9.96 - 11.5)	6.1 - 14.1	0.0073[a]
4	Test Diet	1000	19	10.4 (9.53 - 11.2)	8.2 - 12.3	0.0014[a]
5	Heat-treated Control Diet	1000	26	11.3 ± 1.63[b]	9.6 - 15.0	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Treatment 6 contains a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

[a] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

[b] Standard deviation is provided for mean values not subjected to linear mixed model analysis.

Table 122. Summary Analysis of CMAC IPD072Aa Protein Bioassay Days to Adult Emergence Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	27	14.6 ± 1.95	14	11 - 22	--	--
2	Test Diet	100	28	14.7 ± 1.18	15	13 - 17	0.1529	0.6183
3	Test Diet	500	22	16.6 ± 2.92	16	13 - 23	0.0015[a]	0.3759
4	Test Diet	1000	19	15.9 ± 2.05	16	13 - 20	0.0088[a]	0.1147
5	Heat-treated Control Diet	1000	26	14.3 ± 1.02	14.5	13 - 16	--	--
6	Positive Control Diet	0	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Treatment 6 contains a targeted concentration of 10,000 ng cryolite per mg diet dry weight. Not applicable (NA); there were no surviving *Coleomegilla maculata* in the positive control diet group.

[a] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

XI-D.3. Convergent Ladybird Beetle (CNV)

CNV neonates were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 28 days (Table 131). No adverse effects on survival, weight, or adult emergence of CNV were observed (Table 123, Table 124, and Table 125). The MOE, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 9X the worst-case EEC and 15X the refined for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey (Table 132).

Methods and materials for the bioassay evaluation of CNV are presented in Volume 2, Appendix D, Section D9.

Table 123. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	2	6.67	--
2	Test Diet	1	27[a]	2	7.41	0.6531
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 124. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	19.1 (17.8 - 20.3)	14.3 - 26.2	--
2	Test Diet	1	25	18.0 (16.7 - 19.3)	12.8 - 24.5	0.1157
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 125. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay for Days to Adult Emergence Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Data Points	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel Tukey Test P-Value
1	Bioassay Control Diet	0	28	15.1 ± 0.786	15	13 - 17	--	--
2	Test Diet	1	25	15.2 ± 0.879	15	14 - 17	0.3972	0.6016
3	Positive Control Diet	0[a]	0	NA	NA	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving *H. convergens* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

CNV neonates were exposed to diet containing a target concentration of 100 ng, 500 ng, and 1000 ng IPD072Aa protein/mg diet for 28 days (Table 133) Exposure to 100 ng and 500 ng IPD072Aa protein/mg diet had no adverse effects on survival (Table 126). The MOE, based on a survival NOEC of 500 ng IPD072Aa protein/mg diet, is 13X the worst-case EEC and 31X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey (Table 134).

Sublethal effects were observed on weight of CNV at the 100 ng dose

Acceptability criteria, individual assay results, and materials and methods are presented in Appendix F, Section F8.

Table 126. Summary Analysis of CNV IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	0	0	1.0000
3	Test Diet	500	30	0	0	1.0000
4	Test Diet	1000	30	17[a]	56.7	<0.0001[b]
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0[c]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight.

[a] Two organisms had not emerged as adults by Day 28 of the bioassay and were scored as dead.

[b] A statistically significant difference (P-value < 0.05) was observed.

[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 127. Summary Analysis of CNV IPD072Aa Protein BioassayAdult Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	19.7 (18.9 - 20.4)	16.6 - 23.8	--
2	Test Diet	100	30	18.6 (17.9 - 19.3)	11.6 - 22.6	0.0236[a]
3	Test Diet	500	30	11.3 (10.6 - 12.1)	8.9 - 15.2	<0.0001[a]
4	Test Diet	1000	13	8.63 (7.51 - 9.75)	6.9 - 11.7	<0.0001[a]
5	Heat-treated Control Diet	1000	29	18.5 ± 3.63[b]	11.5 - 25.1	--
6	Positive Control Diet	0[c]	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Standard deviation is provided for mean values not subjected to a t-test.

[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 128. Summary Analysis of CNV IPD072Aa Protein Bioassay Results for Days to Adult Emergence

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	30	13.8 ± 0.664	14	13 - 15	--	--
2	Test Diet	100	30	14.1 ± 0.712	14	13 - 16	0.0551	0.7594
3	Test Diet	500	30	17.4 ± 1.63	17	15 - 21	<0.0001[a]	1.0000
4	Test Diet	1000	13	22.3 ± 2.25	22	19 - 25	<0.0001[a]	0.0015[a]
5	Heat-treated Control Diet	1000	29	14.6 ± 1.50	14	13 - 20	--	--
6	Positive Control Diet	0[b]	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving *Hippodamia convergens* in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

XI-D.4. Parasitic Hymenoptera (*Pediobius foveolatus*)

Parasitic Hymenoptera were exposed to diet containing a target concentration of 1 µg DvSSJ1 dsRNA/ml diet for 14 days (Table 131). No adverse effects on survival were observed (Table 129). The MOE, based on a NOEC of 1 µg DvSSJ1 dsRNA/ml diet, is 9X the worst-case EEC and 15X the refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey (Table 132).

Table 129. Summary Analysis of Parasitic Hymenoptera DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (µg DvSSJ1_210 dsRNA/ml)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	29[a]	5	17.2	--
2	Test Diet	1	30	2	6.67	0.9537
3	Positive Control Diet	0[b]	30	30	100	--

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 8000 µg boric acid per ml.

Parasitic Hymenoptera were exposed to diet containing a target concentration of 100, 500, and 1000 µg IPD072Aa protein/ml diet for 7 days (Table 133). No adverse effects on survival were observed (Table 128). The MOE, based on a NOEC of 1000 µg IPD072Aa protein/ml diet, is 26X the worst-case EEC and 63X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey (Table 134).

Acceptability criteria, individual assay results, and materials and methods are presented in Appendix G, Section G4.

Table 130. Summary Analysis of Parasitic Hymenoptera IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (µg IPD072Aa/ml)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	29[a]	5	17.2	0.0896
3	Test Diet	500	30	5	16.7	0.0973
4	Test Diet	1000	30	6	20.0	0.0514
5	Heat-treated Control Diet	1000	30	5	16.7	--
6	Positive Control Diet	0[b]	30	29	96.7	--

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.
[b] Treatment 6 contained a targeted concentration of 20,000 µg boric acid per ml.

XI-D.5. Predators and Parasitoids – Non-Target Hazard Assessment Conclusions:

Four surrogate species representing the predator and parasitoid functional group were assessed: two Cocinellidae (CMAC and CNV), one Neuroptera (Green Lacewing) and one Hymenoptera (Parasitic Hymenoptera). For the DvSSJ1 dsRNA, no effects on survival were observed in early-tier hazard assessments for any of these surrogate species, after exposure to 9X or 15X the worst-case or refined EECs, respectively. The overall MOE values indicate that DvSSJ1 dsRNA in DP23211 maize is unlikely to be harmful to predators or parasitoids at environmentally realistic concentrations (Table 132).

For the IPD072Aa protein, no effects on survival or pupation were observed for Green Lacewing after exposure to 13X or 31X the worst-case or refined EECs, respectively. Similarly, no effects on survival were observed for Parasitic Hymenoptera, after exposure to 26X or 63X the worst-case or refined EECs, respectively. The overall MOE values for the representative surrogate Neuroptera and Hymenoptera species indicate that the IPD072Aa protein in DP23211 maize is unlikely to be harmful to this predator and parasitoid at environmentally realistic concentrations (Table 134).

Based on the specificity of the IPD072Aa protein, non-target coccinellids are more likely to be sensitive than NTOs from other orders. For CMAC, no effects on survival, weight, or number of days to adult emergence were observed, after exposure to 3X or 6X the worst-case or refined EECs, respectively (Table 133). For CNV, no effects on survival were observed, after exposure to 13X or 31X the worst-case or refined EECs, respectively (Table 133). The MOEs for sub-lethal endpoints (weight and days to adult emergence) for CNV are lower.

As described in the exposure assessment, coccinellid species may feed on pollen or prey, and the predator route was assessed to be most conservative. Several factors will reduce the actual exposure of coccinellid species to the IPD072Aa protein via the predator route, below the worst-case EECs. These factors include: 1) food and prey choice (e.g., not all prey consumed will have consumed DP23211 maize tissue; coccinellid species may feed on pollen or prey); 2) degradation of the IPD072Aa protein in prey; 3) and fresh weight vs. dry weight concentrations (all MOE calculations were conducted based on the dry weight concentration of IPD072Aa protein in DP23211 maize tissues as described in Volume 2, Appendix H). The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017). Therefore, no biologically relevant adverse effects are expected on predators or parasitoids, including non-target Cocinellidae, due to cultivation of DP23211 maize.

XI-E. Insectivorous Birds – Bobwhite Quail

Laboratory bioassay studies were conducted to assess the dietary effect of DvSSJ1 dsRNA and the IPD072Aa protein to insectivorous birds using a suitable surrogate species.

Bobwhite Quail (*Colinus virginianus*) were exposed to a nominal limit dose of 105 mg DvSSJ1 dsRNA/kg body weight for 14 days (Table 131). No mortality, abnormal behavior or signs of toxicity were observed (Table 129 and Volume 2, Appendix E, Section E7). The MOE, based on a NOEL and LD_{50} >105 mg DvSSJ1 dsRNA/kg body weight, is 929X the worst-case EEC and 1,625X the refined EEC for wild birds that are exposed via an insectivorous route of exposure (predator) (Table 132).

Table 131. DvSSJ1_210 dsRNA Northern Bobwhite (*Colinus virginianus*) - Acute Oral Toxicity Test - Survival

Dose	0 mg a.i./kg bw	105 mg a.i./kg bw
Study Day	% Surviving	
0	100	100
1	100	100
2	100	100
3	100	100
4	100	100
5	100	100
6	100	100
7	100	100
8	100	100
9	100	100
10	100	100
11	100	100
12	100	100
13	100	100
14	100	100

Bobwhite Quail were exposed to a nominal limit dose of 2000 mg IPD072Aa protein/kg body weight for 14 days (Table 133). No mortality, abnormal behavior or signs of toxicity were observed (Table 130). The MOE, based on a NOEC and LD_{50} >2000 mg IPD072Aa protein/kg body weight, is 51X the worst-case EEC and 125X the refined EEC for wild birds that are exposed via an insectivorous route of exposure (predator) (Table 134).

Acceptability criteria, individual assay results, and materials and methods are presented in Appendix G, Section G7.

Table 132. Summary of Survival of Northern Bobwhite During the Acute Oral Toxicity Limit Test With Recombinant IPD072Aa protein

Dose (mg/kg body weight)	Cumulative Dead by Study Day		Total # Dead	Total % Mortality
	0 - 7	8 - 14		
0	0	0	0	0
2000	0	0	0	0

XI-E.1. Insectivorous Birds – Non-Target Hazard Assessment Conclusions:

No hazard was detected in early-tier hazard assessments to bobwhite quail after exposure to 929X and 1,625X the worst-case and refined EECs for DvSSJ1 dsRNA (Table 132).

No hazard was detected to bobwhite quail after exposure to 51X or 125X the worst-case and refined EEC for the IPD072Aa protein (Table 134). The overall MOE values indicate that DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize are unlikely to be harmful to insectivorous birds at environmentally realistic concentrations.

XI-F. Granivorous Mammals - Mouse

Based on the low potential for exposure to non-target granivorous mammals, Tier I hazard assessment was not conducted for the DvSSJ1 dsRNA. There are barriers to uptake of dsRNA that likely limit exposure of mammals to the DvSSJ1 dsRNA (VII-A.3.b Mammalian Barriers to Exposure to DvSSJ1 dsRNA Consumed in Food and Feed).

Early tier laboratory studies were conducted to assess the dietary effect of the IPD072Aa protein to non-target granivorous mammals, using a suitable surrogate species.

Mice were orally exposed at a dose of 2000 mg IPD072Aa protein/kg body weight for 14 days (Table 133). No mortality or other evidence of acute oral toxicity was observed, based on evaluation of body weight, clinical signs, and gross pathology. The LD_{50} for Mice was determined to be >2000 mg IPD072Aa/kg body weight. The MOE, based on an LD_{50} >2000 mg IPD072Aa/kg body weight, is 1730X the worst-case EEC for wild mammals exposed via the grain feeding route of exposure (Table 134).

XI-F.1. Granivorous Mammals – Non-Target Hazard Assessment Conclusions:

No hazard is expected for non-target mammals that consume DP23211 maize grain, due to limited potential for exposure to DvSSJ1 dsRNA. Barriers to uptake of dsRNA will likely limit exposure of mammals to the DvSSJ1 dsRNA (VII-A.3.b Mammalian Barriers to Exposure to DvSSJ1 dsRNA Consumed in Food and Feed), and no hazard is expected at environmentally relevant concentrations. No hazard was detected in mice after exposure to 1730X the worst-case EEC for the IPD072Aa protein (Table 134). The overall MOE values for the IPD072Aa protein indicate that the IPD072Aa protein in DP23211 maize are unlikely to be harmful to granivorous mammals at environmentally realistic concentrations.

Table 133. Laboratory Bioassay Studies Characterizing Effects of the DvSSJ1 dsRNA on Representative Non-Target Organisms

Species (Common Name)	Class: Order: Family	Type of study	Concentration	Endpoints assessed	Results
Pollinators and Pollen Feeders					
Apis mellifera (Honey bee larvae)	Insecta: Hymenoptera: Apidae	22-day: DvSSJ1 dsRNA incorporated in diet	Targeted concentration of 0.0040 µg DvSSJ1 dsRNA/larva cell.	Larval survival, pupal survival, adult emergence, adult weight at emergence	No effects on larval survival, pupal survival, adult emergence, or adult weight at emergence were observed. The NOED is 0.0040 µg DvSSJ1 dsRNA/larval cell (equivalent to 4.0 ng DvSSJ1 dsRNA/larvae cell).
Apis mellifera (Honey bee adult)	Insecta: Hymenoptera: Apidae	14-day: DvSSJ1 dsRNA incorporated in diet	Mean daily dose of 0.026 µg DvSSJ1 dsRNA/bee/day	Adult body weight, survival	No effects on adult body weight or survival were observed. The NOEDD is 0.026 µg DvSSJ1 dsRNA/bee/day (equivalent to 26 ng DvSSJ1 dsRNA/bee/day).
Soil-Dwelling Decomposers and Detrivores					
Folsomia candida (Springtail)	Entomobryomorpha: Collembola: Isotomidae	28-day: DvSSJ1 dsRNA incorporated into diet	Targeted concentration of 1 ng DvSSJ1 dsRNA/mg diet	Reproduction, survival	No adverse effect on springtail reproduction or survival were observed. The NOEC is 1 ng DvSSJ1 dsRNA/mg diet

Table 133. Laboratory Bioassay Studies Characterizing Effects of the DvSSJ1 dsRNA on Representative Non-Target Organisms (continued)

Species (Common Name)	Class: Order: Family	Type of study	Concentration	Endpoints assessed	Results
Predators and Parasitoids					
Chrysoperla rufilabris (Green lacewing)	Insecta: Neuroptera: Chrysopidae	21-day: DvSSJ1 dsRNA incorporated into diet	Targeted concentration of 1 ng DvSSJ1 dsRNA/mg diet	Survival, pupation	No adverse effects on survival or pupation of green lacewing were observed. The NOEC is 1 ng DvSSJ1 dsRNA/mg diet
Coleomegilla maculata (Pink spotted lady beetle)	Insecta: Coleoptera: Coccinellidae	28-day: DvSSJ1 dsRNA incorporated into diet	Targeted concentration of 1 ng DvSSJ1 dsRNA/mg diet	Survival, weight, number of days to adult emergence	No adverse effects on survival, weight, or number of days to adult emergence were observed The NOEC is 1 ng DvSSJ1 dsRNA/mg diet
Hippodamia convergens (Convergent ladybird beetle)	Insecta: Coleoptera: Coccinellidae	28-day: DvSSJ1 dsRNA incorporated into diet	Targeted concentration of 1 ng DvSSJ1 dsRNA/mg diet	Survival, weight, adult emergence	No adverse effects on survival, weight, or adult emergence were observed. The NOEC is 1 ng DvSSJ1 dsRNA/mg diet
Pediobius foveolatus (parasitic hymenoptera)	Insecta: Hymenoptera: Eulophidae	14-day: DvSSJ1 dsRNA incorporated into diet	1 µg DvSSJ1 dsRNA/ml diet	Survival	No adverse effect on survival was observed. The NOEC is 1 µg DvSSJ1 dsRNA/ml diet
Insectivorous Birds					
Colinus virginianus (Northern bobwhite quail)	Aves: Galliformes: Odontophoridae	14-day: limit dose of DvSSJ1 dsRNA	Limit dose of 105 mg DvSSJ1 dsRNA/kg body weight	Survival, abnormal behavior, signs of toxicity	No mortality, abnormal behavior or signs of toxicity were observed. The NOEL and LD_{50} are >105 mg DvSSJ1 dsRNA/kg body weight.

Note: median lethal concentration (LD_{50}), no observed effect concentration (NOEC), no observed effect dose (NOED), or no observed effect dietary-dose (NOEDD)

Table 134. Worst-case and Refined Estimated Environmental Concentrations (EEC) and Margin of Exposure (MOE) for Representative Non-Target Organisms Exposed to DvSSJ1 dsRNA from DP23211 Maize

Species (Common Name)	Worst-case EEC	Refined EEC	Hazard Study Result	Margin of Exposure (MOE)[1]
Pollinators and Pollen Feeders				
Apis mellifera (Honey bee larvae)	Larvae eat 2 mg of pollen; Maximum pollen concentration EEC = 4.04×10^{-3} ng/larvae	No refinement to worst-case EEC considered	NOED is 0.0040 µg DvSSJ1 dsRNA/larval cell (equivalent to 4.0 ng DvSSJ1 dsRNA/larvae cell)	990X based on a worst-case EEC.
Apis mellifera (Honey bee adult)	Assume adults consume 4.3 mg of pollen; Maximum pollen concentration. EEC = 8.69×10^{-3} ng/bee	No refinement to worst-case EEC considered	NOEDD is 0.026 µg DvSSJ1 dsRNA/bee/day (equivalent to 26 ng DvSSJ1 dsRNA/bee/day).	2,993X based on a worst-case EEC.
Non-target Lepidoptera	Maximum pollen concentration. EEC = 2.02×10^{-3} ng/mg	No refinement to worst-case EEC considered	Tier I hazard studies on non-target Lepidoptera were not conducted based on negligible potential for exposure. The LC_{50} of DvSSJ1 dsRNA for the most sensitive target pest (WCR) is 0.036 ng/mg.	18X (based on a worst-case EEC for non-target Lepidoptera and the LC_{50} of WCR).
Soil-Dwelling Decomposers and Detritivores				
Folsomia candida (Springtail)	Maximum concentration in senescent (R6) whole plant tissue. EEC = 2.99×10^{-2} ng/mg	Mean concentration in senescent (R6) whole plant tissue. EEC = 1.08×10^{-2} ng/mg	NOEC is 1 ng DvSSJ1 dsRNA/mg diet	33X based on a worst-case EEC. 93X based on a refined EEC.

Table 134. Worst-case and Refined Estimated Environmental Concentrations (EEC) and Margin of Exposure (MOE) for Representative Non-Target Organisms Exposed to DvSSJ1 dsRNA from DP23211 Maize (continued)

Species (Common Name)	Worst-case EEC	Refined EEC	Hazard Study Result	Margin of Exposure (MOE)[1]
Aquatic Organisms				
Aquatic non-target organism	EPA standard pond model (highest mean whole plant concentration across any growth stage). EEC = 2.46×10^{-4} mg/L	No refinement to worst-case EEC considered	Tier I hazard studies on non-target aquatic organisms were not conducted based on negligible potential for exposure. The LC_{50} of DvSSJ1 dsRNA for the most sensitive target pest (WCR) is 0.036 ng/mg.	146X (based on a worst-case EEC for non-target aquatic organism and the LC_{50} of WCR).
Pollinators and Pollen Feeders				
Chrysoperla rufilabris (Green lacewing	Maximum concentration in above-ground tissue (highest expressing tissue across the growing season). EEC = 0.113 ng/mg	Mean concentration in above-ground tissue (highest mean concentration in above ground tissue across the growing season). EEC = 6.46×10^{-2} ng/mg	NOEC is 1 ng DvSSJ1 dsRNA/mg diet	9X based on a worst-case EEC. 15X based on a refined EEC.
Coleomegilla maculata (Pink spotted lady beetle)			NOEC is 1 ng DvSSJ1 dsRNA/mg diet	9X based on a worst-case EEC. 15X based on a refined EEC.
Hippodamia convergens (Convergent ladybird beetle)			NOEC is 1 ng DvSSJ1 dsRNA/mg diet	9X based on a worst-case EEC. 15X based on a refined EEC.
Pediobius foveolatus (parasitic hymenoptera)			NOEC is 1 ng DvSSJ1 dsRNA/mg diet	9X based on a worst-case EEC. 15X based on a refined EEC.

Table 134. Worst-case and Refined Estimated Environmental Concentrations (EEC) and Margin of Exposure (MOE) for Representative Non-Target Organisms Exposed to DvSSJ1 dsRNA from DP23211 Maize (continued)

Species (Common Name)	Worst-case EEC	Refined EEC	Hazard Study Result	Margin of Exposure (MOE)[1]
Insectivorous Birds				
Colinus virginianus (Northern bobwhite quail)	Maximum concentration in above-ground tissue (highest expressing tissue across the growing season). EEC = 0.113 ng/mg	Mean concentration in above-ground tissue (highest mean concentration in above ground tissue across the growing season). EEC = 6.46 x 10^{-2} ng/mg	The NOEL and LD_{50} are >105 mg DvSSJ1 dsRNA/kg body weight.	929X based on a worst-case EEC 1,625X based on a refined EEC.
Granivorous Mammals				
Mus musculus (Mouse)	The worst-case EEC for wild mammals is based on the daily dietary dose (DDD) via consumption of R6 grain. EEC = 2.6 x 10^{-3} mg/kg body weight.	No refinement to worst-case EEC considered	Tier I hazard studies on non-target Granivorous Mammals were not conducted based on negligible potential for exposure.	

[1] all MOEs are rounded to the nearest whole number and are calculated based on tissue dry weight (DW). The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017). Insect bioassays may be reported based on wet weight or dry weight concentrations, and all details related to experimental design are provided in each study in appendices D and E.

Table 135. Laboratory Bioassay Studies Characterizing Effects of the IPD072Aa Protein on Representative Non-Target Organisms

Species (Common Name)	Class: Order: Family	Type of Study	Concentration	Endpoints Assessed	Results
Pollinators and Pollen Feeders					
Apis mellifera (Honey bee larvae)	Insecta: Hymenoptera: Apidae	22-day: IPD072Aa protein incorporated in diet	Targeted concentration of 0.10 and 0.20 µg IPD072Aa protein/larva	Larval survival, pupal survival, adult emergence, adult weight at emergence	No effects on larval survival, pupal survival, adult emergence, or adult weight at emergence were observed. The NOED is 0.20 µg IPD072Aa protein/larval (equivalent to 200 ng IPD072Aa protein/larvae).
Apis mellifera (Honey bee adult)	Insecta: Hymenoptera: Apidae	10-day: IPD072Aa protein incorporated in diet	Mean daily dose of 1.3 µg IPD072Aa protein/bee/day	Adult body weight, survival	No effects on adult body weight or survival were observed. The NOEDD is 1.3 µg IPD072Aa protein/bee/day (equivalent to 1,300 ng IPD072Aa protein/bee/day).
Soil-Dwelling Decomposers and Detritivores					
Folsomia candida (Springtail)	Entomobryomorpha: Collembola: Isotomidae	28-day: IPD072Aa protein incorporated into diet	Targeted concentration of 500 ng IPD072Aa protein/mg diet	Reproduction, survival	No biologically relevant adverse effect on springtail reproduction and no effects on survival were observed. The NOEC is 500 ng IPD072Aa protein/mg diet.Sublethal effect in mean reproduction. The result is considered not to be biologically relevant based on OECD acceptability guidelines and overlapping range of offspring.
Predators and Parasitoids					
Chrysoperla rufilabris (Green lacewing)	Insecta: Neuroptera: Chrysopidae	21-day: IPD072Aa protein incorporated in diet	Targeted concentration of 500 ng IPD072Aa protein/mg diet	Survival, pupation	No adverse effects on survival or pupation of green lacewing were observed The NOEC is 500 ng IPD072Aa protein/mg diet

Table 135. Laboratory Bioassay Studies Characterizing Effects of the IPD072Aa Protein on Representative Non-Target Organisms (continued)

Species (Common Name)	Class: Order: Family	Type of Study	Concentration	Endpoints Assessed	Results
Predators and Parasitoids					
Coleomegilla maculata (Pink spotted lady beetle)	Insecta: Coleoptera: Coccinellidae	28-day: IPD072Aa protein incorporated in diet	Targeted concentration of 100, 500, and 1000 ng IPD072Aa protein/mg diet	Survival, weight, days to adult emergence	Survival NOEC is 100 ng IPD072Aa protein/mg diet. Weight NOEC is 100 ng IPD072Aa protein/mg diet. Emergence NOEC is 1000 ng IPD072Aa protein /mg diet
Hippodamia convergens (Convergent ladybird beetle)	Insecta: Coleoptera: Coccinellidae	28-day: IPD072Aa protein incorporated in diet	Targeted concentration of 100, 500, and 1000 ng IPD072Aa protein/mg diet	Survival, weight, days to adult emergence	Exposure to 100 ng and 500 ng IPD072Aa protein/mg diet had no adverse effects on survival. The survival NOEC is 500 ng IPD072Aa protein/mg diet. Weight effects at all doses.
Pediobius foveolatus (parasitic hymenoptera)	Insecta: Hymenoptera: Eulophidae	7-day: IPD072Aa protein incorporated into diet	Targeted concentration of 100, 500, and 1000 µg IPD072Aa protein/ml diet	Survival	No adverse effect on survival were observed. The NOEC is 1000 µg IPD072Aa protein/ml diet
Insectivorous Birds					
Colinus virginianus (Northern bobwhite quail)	Aves: Galliformes: Odontophoridae	14-day: limit dose of IPD072Aa protein	Limit dose of 2000 mg IPD072Aa protein/kg body weight	Mortality, abnormal behavior, signs of toxicity	No mortality, abnormal behavior or signs of toxicity were observed. The NOEC and the LD_{50} are >2000 mg IPD072Aa protein/kg body weight.

Table 135. Laboratory Bioassay Studies Characterizing Effects of the IPD072Aa Protein on Representative Non-Target Organisms (continued)

Species (Common Name)	Class: Order: Family	Type of Study	Concentration	Endpoints Assessed	Results
Granivorous Mammals					
Mus musculus (Mouse)	Mammalia: Rodentia: Muridae	14-day: Acute oral assay with IPD072Aa protein	Limit dose of 2000 mg IPD072Aa protein/kg body weight	Survival, evidence of acute oral toxicity (based on evaluation of body weight, clinical signs, and gross pathology).	No mortality or other evidence of acute oral toxicity was observed, based on evaluation of body weight, clinical signs, and gross pathology. The LD_{50} is >2000 mg IPD072Aa protein/kg body weight.

Note: median lethal concentration (LD50), no observed effect concentration (NOEC), no observed effect dose (NOED), or no observed effect dietary-dose (NOEDD)

Table 136. Worst-case and Refined Estimated Environmental Concentrations (EEC) and Margin of Exposure (MOE) for Representative Non-Target Organisms Exposed to IPD072Aa Protein from DP23211 Maize

Species (Common Name)	Worst-case EEC	Refined EEC	Hazard study result	Margin of Exposure (MOE) for DP23211 Maize
Pollinators and Pollen Feeders				
Apis mellifera (Honey bee larvae)	Larvae eat 2 mg of pollen; Maximum pollen concentration. EEC = 2.6 ng/mg	No refinement to worst-case EEC considered	NOED is 0.20 µg IPD072Aa protein/larval (equivalent to 200 ng IPD072Aa protein/larvae).	77X based on a worst-case EEC.
Apis mellifera (Honey bee adult)	Assume adults consume 4.3 mg of pollen; Maximum pollen concentration. EEC = 5.59 ng/mg	No refinement to worst-case EEC considered	NOEDD is 1.3 µg IPD072Aa protein/bee/day (equivalent to 1,300 ng IPD072Aa protein/bee/day).	233X based on a worst-case EEC.
Non-target Lepidoptera	Maximum pollen concentration. EEC = 1.3 ng/mg	No refinement to worst-case EEC considered	Tier I hazard studies on non-target Lepidoptera were not conducted based on negligible potential for exposure. The LC_{50} of the IPD072Aa protein for the most sensitive target pest (WCR) is 26 ng/mg	20 X (based on a worst-case EEC for non-target Lepidoptera and the LC_{50} of WCR).
Soil-Dwelling Decomposers and Detritivores				
Folsomia candida (Springtail)	Maximum concentration in senescent (R6) whole plant tissue. EEC = 24 ng/mg	Mean concentration in senescent (R6) whole plant tissue.EEC = 11 ng/mg	NOEC is 500 ng IPD072Aa protein/mg diet	21X based on a worst-case EEC. 45X based on a refined EEC.

Table 136. Worst-case and Refined Estimated Environmental Concentrations (EEC) and Margin of Exposure (MOE) for Representative Non-Target Organisms Exposed to IPD072Aa Protein from DP23211 Maize (continued)

Species (Common Name)	Worst-case EEC	Refined EEC	Hazard study result	Margin of Exposure (MOE) for DP23211 Maize
Aquatic Organisms				
Aquatic non-target *organism*	EPA standard pond model (highest mean whole plant concentration across any growth stage). EEC = 0.124 mg/l	No refinement to worst-case EEC considered	Tier I hazard studies on non-target aquatic organisms were not conducted based on negligible potential for exposure. The LC_{50} of the IPD072Aa protein for the most sensitive target pest (WCR) is 26 ng/mg	210X (based on a worst-case EEC for non-target aquatic organisms and the LC_{50} of WCR).
Predators and Parasitoids				
Chrysoperla rufilabris (Green lacewing)	Maximum concentration in above-ground tissue (highest expressing tissue across the growing season). EEC = 39 ng/mg	Mean concentration in above-ground tissue (highest mean concentration in above ground tissue across the growing season). EEC = 16 ng/mg	NOEC is 500 ng IPD072Aa protein/mg diet	13X based on a worst-case EEC. 31X based on a refined EEC.
Coleomegilla maculata (Pink spotted lady beetle)			NOEC is 100 ng IPD072Aa protein/mg diet	3X based on a worst-case EEC. 6X based on a refined EEC.
Hippodamia convergens (Convergent ladybird beetle)			Survival NOEC is 500 ng IPD072Aa protein/mg diet	13X based on a worst-case EEC. 31X based on a refined EEC.
Pediobius foveolatus (parasitic hymenoptera)			NOEC is 1000 µg IPD072Aa protein/ml diet	26X based on a worst-case EEC. 63X based on a refined EEC.

Table 136. Worst-case and Refined Estimated Environmental Concentrations (EEC) and Margin of Exposure (MOE) for Representative Non-Target Organisms Exposed to IPD072Aa Protein from DP23211 Maize (continued)

Species (Common Name)	Worst-case EEC	Refined EEC	Hazard study result	Margin of Exposure (MOE) for DP23211 Maize
Insectivorous Birds				
Colinus virginianus (Northern bobwhite quail)	Maximum concentration in above-ground tissue (highest expressing tissue across the growing season). EEC = 39 ng/mg	Mean concentration in above-ground tissue (highest mean concentration in above ground tissue across the growing season). EEC = 16 ng/mg	The NOEC and the LD_{50} are >2000 mg IPD072Aa protein/kg body weight.	51X based on a worst-case EEC. 125X based on a refined EEC.
Granivorous Mammals				
Mus musculus (Mouse)	The worst-case EEC for wild mammals is based on the daily dietary dose (DDD) via consumption of R6 grain. EEC = 1.156 mg/kg body weight.	No refinement to worst-case EEC considered	LD_{50} is >2000 mg IPD072Aa protein/kg body weight.	1730X based on a worst-case EEC.

[1] All MOEs are rounded to the nearest whole number and are calculated based on tissue dry weight (DW). The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017). Insect bioassays may be reported based on wet weight or dry weight concentrations, and all details related to experimental design are provided in each study in appendices F and G.

XII. Analysis of Potential Synergism of DvSSJ1 dsRNA and IPD072Aa Protein in DP23211 Maize

Despite the very rare occurrence of biologically relevant synergism between GE insecticidal traits, an assessment for synergism between the DvSSJ1 dsRNA and the IPD072Aa protein was evaluated using the framework outlined by EPA (US-EPA, 2009). There are two different philosophies for how to predict the potency of multiple stressors, as outlined by Borgert et al. (2004). Since the DvSSJ1 dsRNA and the IPD072Aa protein have different modes of action, the independent model of combined action (Bliss, 1939) was used to estimate the potency of a mixture of DvSSJ1 dsRNA and the IPD072Aa protein. WCR, being sensitive to both insecticidal components, was selected to assess the potency of the DvSSJ1 dsRNA and the IPD072Aa protein alone and in combination. First, the medial lethal concentration (LC_{50}) as well as the LC_{10}, LC_{20}, LC_{30}, and LC_{40} of the DvSSJ1 dsRNA alone and the IPD072Aa protein alone were determined. These LC_{50}s were generated over a 14-day exposure duration as the time to effect for DvSSJ1 dsRNA is greater than for IPD072Aa and it is necessary to understand the dose-response profile over the same duration for the synergism assessment. Next, WCR were exposed to diets containing either the LC_{10}, LC_{20}, LC_{30}, LC_{40} or LC_{50} of both the DvSSJ1 dsRNA and the IPD072Aa protein to assess combined potency at each concentration. WCR were also exposed to the single median lethal concentration of DvSSJ1 dsRNA and IPD072Aa protein alone, which provided a measure of how similarly the WCR used in the mixture study performed relative to the WCR used to derive the dose-response profiles of each trait individually. Three separate and independent bioassays were conducted to assess reproducibility of the response of WCR to each mixture. For each bioassay, each diet was provided to 30 individual WCR for a total of 14 days. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After 14 days, each bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality for each treatment was estimated and a 95% confidence interval reported for each estimate.

Results for each bioassay are presented in Table 135 For Treatments 2 through 5, the 95% confidence intervals for estimated mortality encompassed the mortality predicted by the independent model of combined action. For Treatments 6 and 7, the 95% confidence intervals did not contain the expected level of mortality derived from the independent action model and 100% mortality was observed for Treatment 8. Given the observed mortality for treatments 6, 7, and 8 exceeded the mortality predicted by the independent model, additional work focused on quantifying the degree to which the observed mortality was exceeding the expected values. For familiar traits, such as cry proteins, levels of synergism up to 10-fold have been suggested to be acceptable. For traits with less history of familiarity, that acceptance value is decreased to five-fold (US EPA 2009). Therefore, a five-fold increase in potency was considered as a threshold beyond which additional work would be necessary to further characterize synergism.

One way to mathematically define this five-fold threshold is illustrated by a Model Deviation Ratio (MDR) equal to 5 (Belden and Lydy, 2006). That is, the effective concentration of the mixture predicted by the model (independent action in this case), divided by the effective concentration observed from toxicity testing.

To use this threshold based on the MDR, a synergistic effect is equivalent to multiplying the effective concentration of each single active by some factor below the threshold of 5. A factor of 2 was used in this instance to give a hypothetically synergistic mixture demonstrating 2X synergism. This assumption is necessary, as the independent action model for dissimilar modes of action does not assume parallel dose response curves. Multiplying each of the single active concentrations by 2 and then generating expected responses for the single actives and for the mixture allowed for comparison of the observed mortality of the mixtures used in this study against a hypothetical mixture demonstrating 2X synergism (Table 136). The independent model of combined action was then used to generate an expected mortality value for the various mixtures of the two test substances used in this study.

As illustrated in Table 136, the upper 95% confidence limits for observed mortality in Treatments 4-7 are less than the expected mortality for hypothetical mixtures demonstrating 2X synergism. Thus, it can be concluded that the potency of various mixtures of IPD072Aa protein and DvSSJ1 dsRNA are demonstrating less than 2X synergism and well below the 5X threshold considered as necessary for further characterization of the synergistic response. In Treatment 8, observed mortality reached 100% which made comparisons to a hypothetically synergistic mixture impossible. However, WCR demonstrated a dose-dependent response with each of the mixture combinations at lower concentrations and it is reasonable to conclude a lack of a biologically significant synergistic response for this treatment. Further, Belden and Brain (2018) have suggested the independent model of combined action may under-estimate toxicity of mixtures, perhaps due to generalized physiological effects within organisms that are unaccounted for when they are exposed to combinations of stressors. Thus, the independent model may under predict potency of mixtures. Regardless, based on the hypothetical mixture demonstrating 2-fold synergism and the observed WCR mortality there is no biologically relevant synergism with mixtures of IPD072Aa protein and DvSSJ1 dsRNA and significantly less mortality than the established five-fold threshold to require more characterization. Therefore, studies conducted with the single IPD072Aa protein or DvSSJ1 dsRNA are relevant and informative for products containing both traits.

Table 137. Summary analysis of WCR mortality results showing observed and predicted mortalities. Predicted mortalities generated using the independent model of combined action.

Treatment	Treatment Description	Bioassay	Total Number of Observations[a]	Total Number of Dead Organisms	Mortality (%)	Mortality with Abbott's correction (%)	Estimated Mortality (%) with Abbott's Correction (95% Confidence Interval)	Predicted Mortality (%)
1	Bioassay Control Diet [b]	1	28	5	17.9	0	--	0
		2	29	0	0	0		
		3	22	6	27.3	0		
2	LC_{50} of IPD072Aa	1	28	12	42.9	30.4	47.3 (34.3 - 60.7)	50
		2	27	14	51.9	51.9		
		3	20	15	75.0	65.6		
3	LC_{50} of DvSSJ1	1	27	16	59.3	50.4	58.2 (45.0 - 70.3)	50
		2	29	23	79.3	79.3		
		3	25	13	52.0	34.0		
4	LC_{10} of each test substance	1	28	8	28.6	13.0	20.5 (11.9 - 33.0)	19
		2	28	7	25.0	25.0		
		3	25	11	44.0	23.0		
5	LC_{20} of each test substance	1	28	12	42.9	30.4	38.0 (26.2 - 51.4)	36
		2	28	15	53.6	53.6		
		3	23	10	43.5	22.3		
6	LC_{30} of each test substance	1	27	17	63.0	54.9	71.3 (58.6 - 81.4)	51
		2	30	25	83.3	83.3		
		3	29	23	79.3	71.6		
7	LC_{40} of each test substance	1	26	21	80.8	76.6	80.7 (68.1 - 89.1)	64
		2	30	26	86.7	86.7		
		3	22	18	81.8	75.0		
8	LC_{50} of each test substance	1	25	25	100	100	100[c]	75
		2	30	30	100	100		
		3	24	24	100	100		

[a] Organisms counted as missing during the bioassay or lost in transfer, or wells containing more than one organism, were not included in the total number of observations for a given treatment.

[b] Bioassay acceptability criteria is dead and missing organism count $\leqslant$ 30%.

[c] Due to 100% mortality, mortality was not corrected and no confidence interval was generated.

Table 138. Summary of predicted mortality associated with a hypothetical mixture of IPD072Aa protein and DvSSJ1 dsRNA showing 2X synergism and the upper 95% confidence interval for observed mortality of WCR exposed to each treatment.

Treatment ID	Treatment Description	IPD072Aa Protein Concentration	DvSSJ1_210 dsRNA Concentration	2X IPD072Aa Protein Concentration	2X DvSSJ1_210 dsRNA Concentration	Expected Mortality at 2X IPD072Aa Protein Concentration	Expected Mortality at 2X DvSSJ1_210 dsRNA Concentration	Expected Mortality with Mixture at 2X Concentration	Upper 95% Confidence Limit of Observed Mortality for Mixture
4	LC_{10}	6.3	0.0018	12.6	0.0035	42.6	15.6	51.5	33
5	LC_{20}	8.5	0.0054	16.9	0.0107	62.5	29.3	73.5	51.4
6	LC_{30}	10.3	0.011	20.6	0.0225	74.1	41.5	84.9	81.4
7	LC_{40}	12.1	0.021	24.2	0.0412	81.7	52.5	91.3	89.1
8	LC_{50}	14.0	0.036	28.1	0.0719	87.0	62.4	95.1	NA

XIII. Environmental Risk Characterization and Conclusion

Problem formulation was used to develop hypotheses of potential harm to NTOs and the environment, based on knowledge of the receiving environment, the biology of the crop, and the characteristics of the introduced insecticidal traits. Problem formulation was used to guide the exposure and hazard assessments, so that the ERA is informative and predictive of risk (Carstens et al., 2010; Raybould, 2006; Romeis et al., 2013; Wolt and Peterson, 2010).

XIII-A. Biology of the Crop and Receiving Environment

Several characteristics related to the biology of the crop and the receiving environment were considered as part of problem formulation to develop potential pathways to harm related to weediness or outcrossing. The biology of unmodified cultivated maize (*Zea mays* L.), has been described in the documents published by the Organisation for Economic Co-operation and Development (OECD, 2003). These documents contain the information pertaining to aspects of maize biology, including: taxonomy and morphology, use as a crop plant, agronomic practices, center of origin, reproductive biology, potential to be a volunteer weed, possibility and consequences of interspecific and intergeneric crosses with wild relatives, interaction with other organisms, and a summary of ecology.

XIII.A.1. Regions of Maize Cultivation in the United States

Field maize is a major crop worldwide, but represents the largest crop grown in the United States. It is grown in most states, with production concentrated in the Heartland region (including Illinois, Iowa, Indiana, eastern portions of South Dakota and Nebraska, western Kentucky and Ohio, and the northern two-thirds of Missouri). Iowa and Illinois are the top maize-producing states and typically account for slightly more than one-third of the United States crop (USDA-ERS, 2009). Figure 43 indicates acres planted in the United States by county (USDA-NASS, 2011).

Additional maize varieties include popcorn and sweet corn, both of which are minor crops compared to field maize (OECD, 2002). While the range of cultivation of popcorn and sweet maize include the entire United States, in total all acreage represents less than 1% of the acreage of field maize in 2007 (USDA-NASS, 2009).

It is expected that DP23211 maize will be cultivated in the same maize production regions as non-GE, conventional maize.

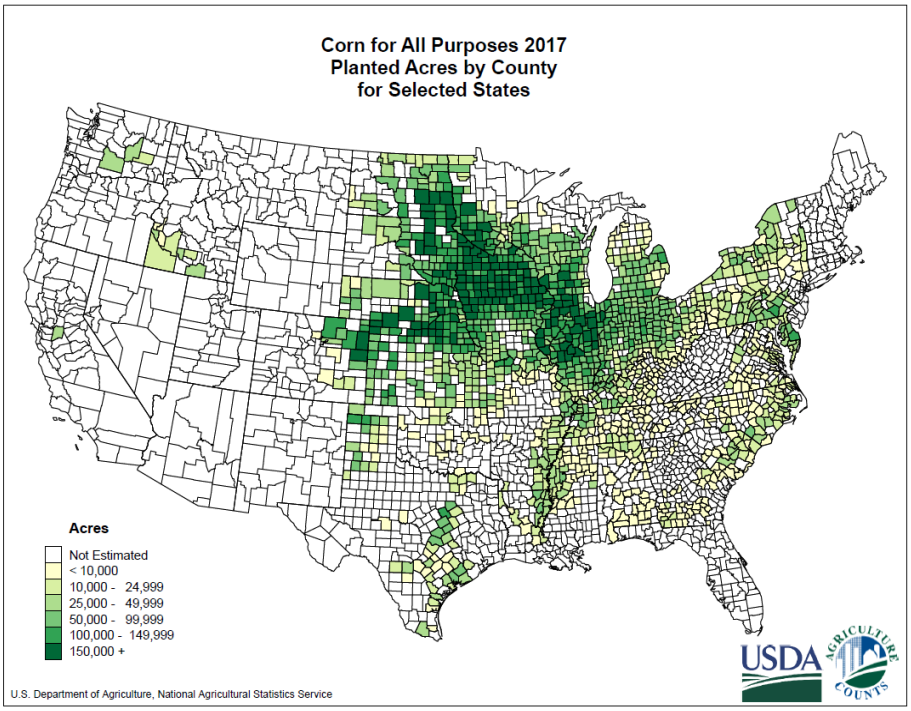


Figure 43. 2017 Corn Planted Acres - USDA-NASS (2017)

XIII-A.1.Potential for Weediness

Maize is extensively cultivated worldwide and has a long history of safe use. Conventional maize is well-established as having low weediness and invasiveness potential, as it is highly domesticated, is unlikely to establish itself in self-sustaining populations outside of cultivation, and is a poor competitor with native vegetation. Maize seeds show poor dormancy (CFIA, 1994) and generally only survive under favorable climatic conditions. Maize is an annual plant that lacks seed dormancy, which limits survival from one growing season to the next (Andersson and de Vicente, 2010; CFIA, 1994). Therefore, the natural characteristics of maize do not indicate a high potential for weediness or invasiveness.

The germination and viability of DP23211 maize was evaluated to determine if there were any differences between DP23211 and a near-isoline control maize (Section VIII-A. Germination and Viability Evaluations). Germination rates in DP23211 maize under warm, cold, and diurnal growing conditions were comparable to those of control maize under corresponding growing conditions. For evaluation of viability, germinated seed were considered viable and ungerminated seed classified as dead were considered non-viable. If ungerminated seed classified as hard or fresh had been identified, a tetrazolium chloride (TZ) test would have been conducted to assess viability; however, no hard or fresh seed were identified.

The data provided here support the conclusion that DP23211 maize is comparable to conventional maize with respect to germination and viability and is unlikely to pose a greater plant pest risk than non-GE, conventional maize.

XIII-A.2.Potential for Gene Flow

The potential for gene flow between a GE crop and its sexually compatible wild relatives is assessed through several factors. One factor includes the potential for pollen flow and outcrossing to occur outside the cultivated field. Other factors include the overlap of the wild relative's geographic distribution with the region of GE crop cultivation and the possibility of genetic compatibility between the crop and the relative. Finally, to determine the potential for widespread introgression of the trait into wild relative populations, the potential for the trait to alter weediness characteristics and the weediness of the wild relative are considered.

Maize has a high outcrossing rate and can pollinate sexually compatible varieties (e.g., other cultivated maize hybrids, teosinte) (OECD, 2003). However, gene flow in the environment is limited by environmental barriers (pollen viability, pollen dispersal, proximity and synchrony of flowering) (Andersson and de Vicente, 2010; CFIA, 1994; Luna et al., 2001; Messeguer et al., 2006) and genetic barriers (ability to outcross and produce fertile progeny) (OECD, 2003).

Maize is almost entirely cross-fertilizing and its pollen is typically wind dispersed (OECD, 2003). Millions of pollen grains are produced per plant (Jarosz et al., 2005). Despite pollination characteristics that are favorable for pollen flow, other factors make it highly unlikely that viable maize pollen will travel significantly outside of the cultivated field. Pollen viability is reduced in a matter of hours under high temperature and low humidity (Aylor, 2004). Studies also indicate that the majority of maize pollen is unlikely to be dispersed significant distances outside the originating field (Jarosz et al., 2003). Numerous studies show the majority (84-92%) of pollen grains travel less than five meters (Pleasants et al., 2001), with nearly all (>99.75%) pollen traveling less than 100 meters (Byrne and Fromherz, 2003; Matsuo et al., 2004; Sears and Stanley-Horn, 2000). The potential of cross-pollination between cultivated maize and its wild relatives will be highest where the wild relatives grow near or adjacent to areas of cultivation. Therefore, the geographic range of wild relatives compared to the regions of maize cultivation is one critical factor in determining the potential for gene flow.

Taxonomically, maize (*Zea mays* L.) is a member of the *Maydeae* tribe of the grass family, *Poaceae* (OECD, 2003). Teosinte, within the genus *Zea*, and the genus *Tripsacum* are the closest relatives to maize taxonomically. The genus *Tripsacum* is also included in the *Maydeae* tribe (OECD, 2003). Annual teosintes are grouped into the species *Zea mays*, although there is some dispute of this classification based on characteristics that prevent a high degree of introgression (OECD, 2003). Annual teosintes have been further classified into the subspecies *Zea mays* ssp. *mexicana* and *Zea mays* ssp. *parviglumis* (OECD, 2003). In contrast, perennial teosintes are

classified as different species altogether: *Zea perennis* and *Zea diploperennis* (OECD, 2003). Both annual and perennial teosintes are considered the closest wild relatives of cultivated maize (OECD, 2003). Perennial plants of the genus *Tripsacum* are considered the next closest relatives of maize (OECD, 2003). Neither the *Zea* genus nor the *Tripsacum* genus are listed as noxious weeds on the federal or state noxious weed lists (USDA-NRCS, 2011).

Both annual and perennial teosintes are normally confined to the tropical and subtropical regions of Mexico, Honduras, Guatemala, and Nicaragua (Iltis, 2011). In the U.S., sparsely dispersed introduced populations of annual teosintes *Zea mexicana* (synonym: *Zea mays* ssp. *mexicana*) and *Zea mays* ssp. *parviglumis* have been reported in Florida, Maryland, and Alabama (USDA, 2011). Also, an isolated population of *Zea perennis* (perennial teosinte) has been introduced in South Carolina (USDA, 2011). While maize can hybridize with these species under natural conditions, there is incompatibility between some maize populations and certain types of teosinte that results in low fitness of some hybrids and prevents a high rate of introgression (OECD, 2003). Together with the very limited geographic range of the teosinte population in the U.S., the probability of gene flow from cultivated maize fields to these wild relatives is very low.

Plants of the genus *Tripsacum* are mostly found in Mexico, Central, and South America (OECD, 2003). Three of these species (*T. dactyloides, T. floridanum*, and *T. lanceolatum*) exist as native species populations in the continental U.S., and two species (*T. fasciculatum* and *T. latifolium*) were introduced in Puerto Rico (USDA, 2011). *T. dactyloides* occurs throughout the eastern half of the U.S. *T. lanceolatum* occurs in Arizona and New Mexico (USDA, 2011) and *T. floridanum* is native to southern Florida (USDA, 2011). Although it is extremely difficult, *Tripsacum* species (*T. dactyloides*, *T. floridanum*, and *T. lanceolatum*) can be crossed with maize; however, hybrids have a high degree of sterility and are genetically unstable (OECD, 2003). Successful crosses of maize with *Tripsacum* species have been made experimentally, however such crosses are not known to occur in the wild (OECD, 2003). Therefore, gene flow between cultivated maize and relatives of the genus *Tripsacum* is highly unlikely.

No significant differences were observed between DP23211 maize and conventional maize in pollen viability via measurements of shape and color over time (VIII-B.1. Agronomic Evaluation of DP23211 Maize). Pollen viability of DP23211 maize is comparable and no difference in pollination biology is expected when compared to conventional maize. Therefore, based on pollen viability data for DP23211 maize, it is unlikely that DP23211 would pose a greater plant pest risk than conventional, non-GE maize.

XIII-B. Characteristics of the Introduced Insecticidal Traits

Several characteristics related to the introduced insecticidal traits were considered as part of problem formulation (e.g., insecticidal modes of action, potential for interaction between the insecticidal traits, similarity with previously assessed traits, specificity of the traits). These characteristics are discussed further below for DvSSJ1 dsRNA and the IPD072Aa protein expressed by DP23211 maize.

XIII-B.1. Insecticidal Modes of Action

DP23211 plants express DvSSJ1 dsRNA, which is intended to down-regulate expression of the DvSSJ1 protein in the mid-gut of the WCR via RNAi. The DvSSJ1 dsRNA is targeted to match the sequence of the smooth septate junction protein 1 (*dvssj1*) gene from WCR. Smooth septate junctions (SSJ) are unique to invertebrates and are composed of a network of proteins that physically connect adjacent cells (IX-B.1. DvSSJ1 dsRNA Mode of Action and Molecular Target). The physical integrity of the SSJ is important for controlling the paracellular pathway between epithelial cells, which separates the gut lumen where digestion occurs from the interstitial space where metabolites and electrolytes are tightly regulated. When DP23211 plants expressing DvSSJ1 dsRNA are ingested by WCR, production of the DvSSJ1 protein in the intestinal lining is suppressed. Reduction in the DvSSJ1 protein, and the subsequent loss of the gut epithelial barrier and cellular deformities, is lethal to WCR (Hu *et al.*, 2016; Hu *et al.*, 2019). The DvSSJ1 dsRNA has been shown to be highly specific, with activity limited to species within the genus *Diabrotica* and family Chrysomelidae (IX-B.2. DvSSJ1 dsRNA Spectrum of Activity and Species Specificity).

The IPD072Aa protein, encoded by the *ipd072Aa* gene, provides control of corn rootworms (*Diabrotica spp*.) when expressed in plants. The IPD072Aa protein is a non-pore forming protein that has a midgut site of action (SoA) where it targets and disrupts midgut epithelial cells causing breakdown of the epithelial lining. Specific binding of IPD072Aa to midgut epithelial cells was confirmed using competitive binding assays with brush border membrane vesicles (BBMVs) prepared from WCR midgut tissue. Binding of the IPD072Aa protein to WCR BBMVs did not occur under alkaline conditions, which mimic the lepidopteran midgut environment, and was also absent when using BBMVs prepared from ECB midgut tissue. These results are consistent with the necessity for a specific receptor interaction in WCR, that is responsible for its insecticidal activity. These results are also consistent with the lack of that specific interaction or bioactivity of IPD072Aa protein against lepidopteran insects (IX-C.1. IPD072Aa Mode of Action). The IPD072Aa protein has been shown to be specific, with activity limited to within the order Coleoptera (IX-C.2.a. Feeding Bioassay Assessment of IPD072Aa Protein Specificity).

XIII-B.2. Potential for Interaction between DvSSJ1 dsRNA and the IPD072Aa Protein

The potential for interaction, as it relates to synergism, between the DvSSJ1 dsRNA and the IPD072Aa protein was evaluated using the framework outlined by EPA (US-EPA, 2009) (see

Section XII. Analysis of Potential Synergism of DvSSJ1 dsRNA and IPD072Aa Protein in DP23211 Maize). As described above, the DvSSJ1 dsRNA and the IPD072Aa protein have different modes of action. Therefore, the independent model of combined action (Bliss, 1939) was used to estimate the potency of a mixture of DvSSJ1 dsRNA and the IPD072Aa protein (XI. Analysis of Potential Synergism of DvSSJ1 dsRNA and IPD072Aa Protein in DP23211 Maize). The results showed no evidence of biologically relevant synergistic effects between the DvSSJ1 dsRNA and the IPD072Aa protein, based on the EPA framework (US-EPA, 2009). Thus, safety studies conducted individually on the DvSSJ1 dsRNA or the IPD072Aa protein are appropriate to be used to inform the ERA of DP23211 maize.

XIII-B.3. Similarity to Previously Assessed Traits

RNA interference (RNAi) is a naturally occurring mechanism for down-regulation of gene expression in most plants and animals, and it involves a process that promotes an RNA

transcripts degradation when dsRNA is endogenously transcribed or exogenously introduced into a cell with a sequence that is complementary to the mRNA produced by transcription of a gene. There are many reviews that describe the RNAi machinery and provide a detailed mechanism of action of RNAi (for example, Fire *et al.*, 1998; Kurreck, 2009; Mello and Conte, 2004; Price and Gatehouse, 2008). Several GM crops have, or are being developed using RNAi to improve taste or nutritional profile, provide resistance to viruses, and to control of insect pests (for example, Anderson et al., 2016; Baum et al., 2007; Bonfim et al., 2007; Krieger et al., 2008; Mao et al., 2011; Pavely et al., 2007). Therefore, the use of RNAi in the field of agricultural biotechnology has a history of safety, and the established ERA framework for GM crops is robust and suitable for assessing plants developed using RNAi (CERA, 2011a). The DvSSJ1 trait produces dsRNA, which down-regulates expression of the DvSSJ1 protein in the mid-gut of the WCR. The DvSSJ1 dsRNA has been shown to be highly specific, with activity limited to species within the genus *Diabrotica* and the family Chrysomelidae.

The IPD072Aa protein is derived from *Pseudomonas chlororaphis* (Schellenberger et al., 2016). *P. chlororaphis* is a naturally occurring, ubiquitous bacterium found in the environment that lacks known allergenic or toxic properties and has a history of safe use in agriculture (Anderson et al., 2018). Certain *Pseudomonas* species, including *P. chlororaphis,* have been used in agriculture as seed treatments, foliar-applied biopesticides for fungal and disease control, and as a gene source for GM crops. Over the past 30 years, the US EPA has registered several *Pseudomonas*-based biopesticides and granted exemptions from the requirements of a tolerance (e.g. 40 CFR 180-1114, 180.1145, 1802.1212) further demonstrating the history of safe use with this source organism (Anderson et al., 2018). The established ERA framework that is currently used to assess GM crops derived from *Bt* is robust and suitable for assessing plants expressing non-*Bt* proteins

(Anderson et al., 2018). The IPD072Aa protein has been shown to be specific, with activity limited to within the order Coleoptera.

XIII-F. Environmental Risk Assessment of DP23211 Insecticidal DvSSJ1 dsRNA and IPD072Aa Protein

The ERA of DP23211 maize focused on the insecticidal DvSSJ1 dsRNA and the IPD072Aa protein expressed in DP23211 maize and assessed the potential exposure and hazard to NTOs. Potential pathways to harm related to weediness and outcrossing were also considered.

Worst-case EECs of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize were determined using worst-case assumptions to determine potential exposure of NTOs. Several factors that reduce potential exposure to NTOs under more realistic environmental conditions were considered and used to refine EECs when needed to understand environmentally relevant concentrations. Early-tier laboratory toxicity studies were performed using the DvSSJ1 dsRNA and the IPD072Aa protein individually, given the demonstrated lack of biologically relevant synergism and independent modes of action. Environmental risk was characterized by comparing Tier I hazard study results to worst-case EECs or refined EECs to calculate the MOEs for the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize.

Based on the concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen, the worst-case exposure of non-target pollinators and pollen feeders to the DvSSJ1 dsRNA and IPD072Aa protein is considered negligible, as these exposures are lower than the LC_{50} concentrations of the most sensitive target pest, WCR. Several additional factors will further reduce the exposure of non-target pollinators and pollen feeders to DvSSJ1 dsRNA and the IPD072Aa protein below the worst-case EECs (XI-A. Pollinators and Pollen Feeders).

No hazard was detected in the early-tier hazard assessment of a representative non-target detritivore after exposure to 33X and 21X the worst-case EEC of the DvSSJ1 dsRNA and IPD072Aa protein, respectively. The refined EECs were even higher and represent more realistic environmental exposures. Based on the overall MOE values, the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize are unlikely to be harmful to non-target soil dwelling organisms at environmentally realistic concentrations (XI-B. Soil-Dwelling Organisms).

Tier I hazard studies on aquatic species were not conducted based on negligible potential for exposure to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize. The DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize is unlikely to be harmful to non-target aquatic organisms at environmentally realistic concentrations (X-.C. Aquatic Non-Target Organisms).

Four surrogate species representing the predator and parasitoid functional group were assessed: two Cocinellidae (CMAC *and* CNV), one Neuroptera (*C. rufilabris)* and one Hymenoptera (*P.*

foveolatus). For the DvSSJ1 dsRNA, no effects on survival were observed in early-tier hazard assessments for any of these surrogate species, after exposure to 9X or 15X the worst-case or refined EECs, respectively. The overall MOE values indicate that DvSSJ1 dsRNA in DP23211 maize is unlikely to be harmful to predators or parasitoids at environmentally realistic concentrations. For the IPD072Aa protein, no effects on survival or pupation were observed for *C. rufilabris,* after exposure to 13X or 31X the worst-case or refined EECs, respectively. Similarly, no effects on survival were observed for *P. foveolatus,* after exposure to 26X or 63X the worst-case or refined EECs, respectively. The overall MOE values for the representative surrogate Neuroptera and Hymenoptera species indicate that the IPD072Aa protein in DP23211 maize is unlikely to be harmful to at environmentally realistic concentrations (XI-D.1. Green Lacewing (*Chrysoperla rufilabris)*).

Based on the specificity of the IPD072Aa protein, non-target coccinellids are more likely to be sensitive than NTOs from other orders. For CMAC*,* no effects on survival, weight, or number of days to adult emergence were observed, after exposure to 3X or 6X the worst-case or refined EECs (VI-B.4.a.9. Pink spotted lady beetle (*Coleomegilla maculata*)), respectively. For *CNV,* no effects on survival were observed, after exposure to 13X or 31X the worst-case or refined EECs, respectively. The MOEs for sub-lethal endpoints (weight and days to adult emergence) for CNV are lower. However, as described in the exposure assessment, coccinellid species may feed on pollen or prey, and the predator route was assessed to be most conservative (VII.A.3. Non-target Coccinellids). Several factors will reduce the exposure of non-target coccinellids to the IPD072Aa protein via the predator route below the worst-case EECs. These factors include: 1) food and prey choice (i.e., not all prey consumed will have consumed DP23211 maize tissue); 2) degradation of the IPD072Aa protein in prey (the IPD072Aa protein will likely not persist in prey); 3) and fresh weight vs. dry weight concentrations (all MOE calculations were conducted based on the dry weight concentration of IPD072Aa protein in DP23211 maize tissues). The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017).

The DvSSJ1 dsRNA has been shown to be highly specific, with activity limited to within the family Chrysomelidae (VI-A.4. DvSSJ1 dsRNA Spectrum of Activity and Species Specificity), and the IPD072Aa protein has been shown to be specific, with activity limited to within the order Coleoptera (VI-B.4. IPD072Aa Spectrum of Activity and Species Specificity) The MOE values for DP23211 maize indicate that the DvSSJ1 dsRNA and the IPD072Aa protein are not expected to be harmful to NTO populations at environmentally relevant concentrations (Table 132 and Table 134). Furthermore, based on what is known about the basic biology of maize, the receiving environment, the intended trait, the risk of increased weediness or outcrossing is expected to be low for DP23211 maize.

XIII-G. Environmental Risk Conclusions

In conclusion, no adverse effects to NTO populations are expected as a result of cultivation of DP23211 maize based on the expected levels of exposure to the DvSSJ1 dsRNA and the IPD072Aa protein and the results of Tier I laboratory toxicity studies.

The analysis of toxicity and specificity of the PIP and RNAi, exposure to sensitive nontarget organisms beneficial to the agricultural environment of the GE plants, potential for interaction, similarity to previously assessed traits, and the peer reviewed literature demonstrate that exposure to and/or consumption of DP23211 maize and the expressed PIPs are unlikely to have any direct or indirect adverse impacts on species beneficial to agriculture, and unlikely to pose a plant pest risk.

XIV. Potential Environmental Impact of the Introduction of DP23211 Maize

The potential environmental impact of a GE plant needs to be considered in the context of the characteristics of the recipient crop, the introduced trait, and the environment in which it will be introduced (OECD, 1993). Knowledge in each of these areas will provide background on which a risk or safety assessment can be made about the environmental release of the GE plant (OECD, 1993). Weediness, gene transfer or flow, and trait effects are particular issues that may be relevant to evaluating the new GE line and its safety (OECD, 1993).

To evaluate the potential environmental impact of the introduction of DP23211 maize, the potential for DP23211 maize to become weedy or invasive, the potential for gene flow to sexually compatible wild relatives, and the potential impacts of the introduced DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI proteins were considered. As described further below, in each case, it is not expected that DP23211 maize will adversely impact the environment with respect to these considerations.

XIV-A. Potential for DP23211 Maize to Have Altered Disease and Unintended Pest Susceptibilities or to Become Weedy or Invasive

In evaluating the potential for DP23211 maize to become more weedy or invasive than conventional maize, general maize biology was considered. Maize is a cultivated annual plant that generally cannot survive temperatures below freezing and is typically grown in temperate regions (OECD, 2003). Maize is not classified as a weed, is not on the United States federal or state noxious weed lists, and possesses few characteristics of notably successful weeds (Baker, 1974; Keeler, 1989; USDA-NRCS, 2011). Therefore, the natural characteristics of maize do not indicate a high potential for weediness or invasiveness.

A comparative assessment of DP23211 maize was conducted to determine if the DNA insertion altered the agronomic characteristics of maize. Agronomic comparison data were collected on DP23211 maize in multiple location field trials as described in Section VIII. Agronomic Performance Assessment and Ecological Observations. This analysis showed that DP23211 maize was comparable to conventional maize and was comparable in agronomics. In the agronomic analyses, plant characteristics were measured, including certain endpoints that may be indicative of weediness: germination and emergence (germination rate, early stand count); reproductive characteristics (days to flowering, days to maturity, pollen viability, dropped ears, yield, and 100-kernel weight); vegetative characteristics (final population, lodging, plant height); and pest response (abiotic and biotic stressors). Characteristics related to seed germination, seed production, reproductive time and vegetative competitiveness have been identified with successful weeds (Baker, 1974). Changes to these parameters relative to the conventional variety

could indicate a change in the potential weediness of a crop. DP23211 maize was comparable to conventional maize in each of these characteristics, indicating that DP23211 maize is unlikely to become more weedy or invasive than conventional maize.

In addition, DP23211 maize has been field tested over multiple years in multiple locations that provide a range of environmental conditions and include regions representative of maize cultivation in the United States. These fields were frequently monitored by expert growers for the incidence of diseases and insects and the effect of these on DP23211 maize and control plants. In all cases, no unexpected differences were observed between DP23211 maize and the control comparators.

In summary, DP23211 maize is unlikely to become more weedy or invasive than conventional maize when cultivated. Agronomic comparisons indicate no unexpected effects of the presence of the introduced proteins that alter the nutritional composition and weediness potential of maize. No unexpected differences were detected between DP23211 maize and control maize in response to insects and diseases. Furthermore, the expression of the introduced DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI proteins is unlikely to increase the potential of DP23211 maize to become weedy.

XIV-B. Potential for Gene Flow Between DP23211 Maize and Sexually Compatible Wild Relatives

The potential for gene flow between a GE crop and its sexually compatible wild relatives is assessed through several factors. One factor includes the potential for pollen flow and outcrossing to occur significantly outside the cultivated field. Other factors include the overlap of the wild relative geographic distribution with the region of GE crop cultivation and the possibility of genetic compatibility between the crop and the relative. Finally, to determine the potential for widespread introgression of the trait into wild relative populations, whether the trait itself alters weediness characteristics and whether the wild relative is a noxious weed is considered.

DP23211 maize is anticipated to be cultivated similarly to other conventional maize varieties; therefore, it is appropriate to examine maize pollination biology, regions of maize cultivation in the United States and the geographic distribution of sexually compatible wild relatives to determine the potential for gene flow. The regions of maize cultivation in the United States and the genetic compatibility and geographic distribution of sexually compatible wild relatives of maize, within the genera *Zea* and *Tripsacum*, are discussed further below. Based on this information, there is low potential for gene flow between DP23211 maize and its wild relatives of the genera *Zea* and *Tripsacum* in the United States.

The potential for the insertion in DP23211 maize to become widespread in wild relative populations is also unlikely. The insertion does not make DP23211 maize more weedy than conventional cultivated maize; furthermore, none of the sexually compatible wild relatives are listed as noxious weeds.

XV. Adverse Consequences of Introduction

The data and information presented in this petition demonstrate that DP23211 maize is unlikely to pose a plant pest risk as compared to conventional maize. The analysis of molecular data confirmed the insertion of a single copy of the intended insertion, containing the DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI protein expression cassettes. The intended insert is stably integrated at a single locus and follows Mendelian inheritance principles over multiple breeding generations.

The analysis of nutrients, anti-nutrients, and secondary metabolites of DP23211 maize demonstrates the compositional comparability of DP23211 maize to conventional maize. Evaluation of seed germination and viability characteristics showed comparability to conventional maize controls. Agronomic characteristics inclusive of plant growth and development, reproductive, and vegetative parameters were comparable to non-GE conventional maize.

Measurement of response to biotic and abiotic stressors, insects, or disease also show comparability when compared to conventional maize. This dataset supports the conclusion that DP23211 maize is unlikely to have an adverse impact on non-target or beneficial organisms.

Introduction of DP23211 maize is not anticipated to impact cultivation practices, including the management of insects, weeds, or diseases in current maize production.

No adverse effects to NTO populations are expected as a result of cultivation of DP23211 maize based on the expected levels of exposure to the DvSSJ1 dsRNA and the IPD072Aa protein and the results of laboratory studies.

The data and information contained herein supports the conclusion that DP23211 maize does not present a greater plant pest risk than non-GE conventional maize varieties and is not otherwise deleterious to the environment. Therefore, Pioneer requests that APHIS grant the request for a determination of nonregulated status for DP23211 maize, DP23211 maize progeny, and any crosses of DP23211 maize with other nonregulated maize.

Appendices

Appendix 1. DP23211 Maize USDA Release Permits, Notifications, and Planted Acreage

Year	Permit Name	Permit Valid Date	State	County	Acreage	Number of Plantings
2015	15-096-101n	4/27/2015	PR	Salinas	0.001	1
	15-309-101n	12/7/2015	HI	Kauai	0.002	1
2016	16-039-106n	3/1/2016	HI	Kauai	0.002	1
	16-293-101rm-a1	11/10/2016	HI	Kauai	0.008	4
			PR	Salinas	0.153	4
2017	17-038-105rm-a1	3/21/2017	IL	Bureau	0.002	1
				Champaign	0.001	1
				McDonough	0.002	1
			IN	Tipton	0.002	1
			IA	Dallas	0.001	1
				Polk	0.013	2
			NE	York	0.002	1
			TN	Obion	0.001	1
	17-264-103rm	11/6/2017	HI	Kauai	0.097	6
	17-311-102rm	11/30/2017	PR	Salinas	0.055	1
	17-264-103rm	11/6/2017	HI	Kauai	0.017	1
2018	18-016-102rm -	3/13/2018	IN	Clinton	0.011	1
				Shelby	0.011	1
			IA	Greene	0.011	1
				Jefferson	0.011	1
				Shelby	0.011	1
			KS	Pawnee	0.011	1
			MN	Freeborn	0.023	1
			MO	Adair	0.011	1

DP23211 Maize USDA Release Permits, Notifications, and Planted Acreage (cont'd)

Year	Permit Name	Permit Valid Date	State	County	Acreage	Number of Plantings
2018	18-016-102rm -	3/13/2018	MO	Butler	0.011	1
			NE	York	0.104	4
			NJ	Hunterdon	0.011	1
			OK	Marshall	0.011	1
			PA	Lehigh	0.011	1
			TX	Armstrong	0.011	1
				Tom Green	0.011	1
			WA	Grant	0.028	1
			WI	Walworth	0.011	1
	18-033-102rm	4/5/2018	CA	Yolo	0.007	2
			IL	Bureau	0.002	1
				Champaign	0.019	3
				Coles	0.001	1
				Piatt	0.002	1
			IN	Benton	0.002	1
				Newton	0.002	1
				Tipton	0.021	2
			IA	Bremer	0.002	1
				Jasper	0.001	1
				Linn	0.003	2
				Polk	0.057	6
			MN	Blue Earth	0.021	2
				Swift	0.002	1
			NE	Platte	0.002	1
				York	0.028	3
			SD	Brookings	0.002	1
			TN	Obion	0.001	1
			TX	Hale	0.007	2
			WI	Rock	0.002	1
	18-033-103rm	4/5/2018	CA	Yolo	0.017	5
			IL	Bureau	0.008	2
				Champaign	0.004	1
				Coles	0.004	1
			IN	Tipton	0.004	1
			IA	Jasper	0.004	1
				Linn	0.003	1

DP23211 Maize USDA Release Permits, Notifications, and Planted Acreage (cont'd)

Year	Permit Name	Permit Valid Date	State	County	Acreage	Number of Plantings
2018	18-033-103rm	4/5/2018	IA	Madison	0.004	1
				Polk	0.008	3
			KS	Hodgeman	0.01	3
			MO	Scott	0.004	1
			NE	Lancaster	0.004	1
				York	0.008	2
			TN	Obion	0.004	1
			TX	Hale	0.01	3
	18-102-102rm -	5/23/2018	IL	Madison	0.065	2
			IN	Clinton	0.011	1
			IA	Howard	0.011	1
			SD	Brookings	0.011	1
	18-192-107rm	11/1/2018	HI	Kauai	0.017	2
			PR	Salinas	0.011	1
	18-192-110rm	11/1/2018	HI	Kauai	0.026	5
			PR	Salinas	0.119	8
	18-192-110rm	11/1/2018	PR	Salinas	0.002	1
2019	19-028-115rm	3/27/2019	MD	Kent	0.018	1
	19-030-101rm	3/29/2019	IL	Bureau	0.002	1
				Champaign	0.005	2
			IN	Tipton	0.002	1
			IA	Linn	0.005	2
				Polk	0.018	6
				Poweshiek	0.001	1
			MN	Blue Earth	0.003	1
			NE	York	0.005	2
			SD	Brookings	0.003	1
			TN	Obion	0.001	1
			WI	Rock	0.001	1

Appendix 2. Methods and Results for Southern by Sequencing Analysis

Seeds from the T1 generation of DP23211 maize were planted, and leaf tissue harvested.

Control Material

Seeds from an unmodified maize line, PHR03, were planted and leaf tissue harvested from individual plants was used in genomic DNA extraction.

Reference Material

Plasmids PHP74643, PHP56614, PHP21139, and PHP31729 were used as positive controls to show successful capture of targeted sequences by the biotinylated probes used in SbS.

Plant Growth and Sample Collection

Test and control substance (DP23211 maize and control maize) seeds were planted and grown and leaf tissue was collected. The leaf samples used for DNA extraction and SbS analysis were maintained frozen (≤ -50 °C) until processing.

DNA Extraction and Quantification

Genomic DNA was extracted from leaf tissue of DP23211 and control maize plants. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep) and the genomic DNA was isolated using a standard Urea Extraction Buffer procedure. Following extraction, the DNA was quantified on a spectrofluorometer using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes, Inc.) and visualized on an agarose gel to determine the DNA quality.

Southern by Sequencing

SbS was performed by Pioneer Genomics Technologies. SbS analysis utilizes probe-based sequence capture, Next Generation Sequencing (NGS) techniques, and bioinformatics procedures to capture, sequence, and identify inserted DNA within the maize genome (Zastrow-Hayes et al., 2015). By compiling a large number of unique sequencing reads and mapping them against the linearized transformation plasmid map and control maize genome, unique junctions due to inserted DNA are identified in the bioinformatics analysis. This information was used to determine the number of insertions within the plant genome, verify insertion intactness, and confirm the absence of plasmid backbone sequences. The T1 generation of DP23211 maize was analyzed by SbS to determine the insertion copy number and intactness. SbS was also performed on control maize DNA and positive control samples (control maize DNA spiked with plasmid DNA at a level corresponding to one copy of plasmid per copy of the maize genome) to confirm that the assay could reliably detect plasmid fragments within the genomic DNA.

The following processes were performed by Pioneer Genomics Technologies using standard methods, and were based on the procedures described in Zastrow-Hayes et al. (2015).

Capture Probe Design and Synthesis

Biotinylated capture probes used to select plasmid sequences were designed and synthesized by Roche NimbleGen, Inc. The probe set was designed to target all sequences within the PHP74643, PHP56614, PHP21139, and PHP31729 plasmids (Figure 10, Step 2).

Sequencing Library Construction

Next generation sequencing (NGS) libraries were constructed for DNA samples from individual DP23211 maize plants, a control maize plant, and the positive control samples. Genomic DNA purified as described above was sheared to an average fragment size of 400 bp using an ultrasonicator. Sheared DNA was end-repaired, A-tailed, and ligated to NEXTflex-HT™ Barcode adaptors (Bioo Scientific Corp.) following the kit protocol so that samples would be indexed to enable identification after sequencing. The DNA fragment libraries were amplified by PCR for eight cycles prior to the capture process. Amplified libraries were analyzed using a fragment analyzer and diluted to 5 ng/µl with nuclease-free water (Figure 10, Step 3).

Probe Hybridization and Sequence Enrichment

A double capture procedure was used to capture and enrich DNA fragments that contained sequences homologous to the capture probes. The genomic DNA libraries described above were mixed with hybridization buffer and blocking oligonucleotides corresponding to the adapter sequences and denatured. Following denaturation, the biotinylated probes were added to the genomic DNA library and incubated at 47 °C for 16 hours. Streptavidin beads were added to the hybridization mix to bind DNA fragments that were associated with the probes. Bound fragments were washed and eluted, PCR-amplified for five cycles, and purified using spin columns. The enriched DNA libraries underwent a second capture reaction using the same conditions to further enrich the sequences targeted by the probes. This was followed by PCR amplification for 16 cycles and purification as described above. The final double-enriched libraries were quantified and diluted to 2 nM for sequencing (Figure 10, Step 4).

Next Generation Sequencing on Illumina Platform

Following sequence capture, the libraries were submitted for NGS to a depth of 100x for the captured sequences. The sequence reads were trimmed for quality below Q20 (Ewing and Green, 1998; Ewing et al., 1998) and assigned to the corresponding individual plant based on the

indexing adapters. A complete sequence set from each plant is referred to as "AllReads" for bioinformatics analysis of that plant (Figure 10, Step 5).

Quality Assurance of Sequencing Reads

The adapter sequences were trimmed from the NGS sequence reads with custom scripts. Further analysis to eliminate sequencing errors used JELLYFISH, version 1.1.4 (Marçais and Kingsford, 2011), to exclude any 31 bp sequence that occurred less than twice within "AllReads" as described in Zastrow-Hayes et al. (2015). This set of sequences was used for further bioinformatics analysis and is referred to as "CleanReads". Identical sequence reads were combined into non-redundant read groups while retaining abundance information for each group. The read group sequences from the most abundant 60% of the non-redundant groups (referred to as "Non-redundantReads") were used for further analysis, as described in Zastrow-Hayes et al. (2015) (Figure 10, Step 6).

Filtering Reads

Each set of "Non-redundantReads" was aligned to the maize reference genome using Bowtie, version 1.0.0 (Langmead et al., 2009) with up to two mismatches allowed. The "Non-redundantReads" not matching the maize reference genome were then compared to the plasmid sequences T-DNA sequence using Bowtie with zero mismatches allowed. Any "Non-redundantReads" that were not wholly derived from either sequence were aligned maize or plasmid backbone sequences with Bowtie 2, version 2.1.0, allowing zero mismatches. The ubiquitous presence of environmental bacteria, such as *Serratia marcescens,* provides an opportunity for their plasmid DNA to be sequenced along with plant genomic DNA. This resulted in low level detection of plasmid backbone sequences in the genomic DNA samples due to similarity with the plasmid backbone region. "Non-redundantReads" that aligned to the plasmid backbone sequence, but at a coverage depth below 35x across 50 bp, were deemed to be due to environmental bacteria (Figure 10, Step 7). Due to the detection of these bacterial sequences, coverage levels of 35x or below were considered to be the background level of sequencing.

Junction Detection

Following removal of "Non-redundantReads" with alignments wholly to the maize reference genome or plasmid sequence identified during the quality assurance phase, the remaining "Non-redundantReads" were aligned to the full plasmid sequence using BWA, version 0.5.9-r16, with the soft-trimming feature enabled (Li and Durbin, 2010). Chimeric reads contain sequence that is non-contiguous with the plasmid sequence from the alignment, such as plasmid-to-genome junctions or rearrangements of the plasmid. These chimeric reads are referred to as junction reads or junctions. The individual reads defining a junction were condensed to a unique identifier to represent the junction. This identifier (referred to as a 30_20 mer) includes 20 bp of sequence

from PHP74643, PHP56614, PHP21139, or PHP31729, and 30 bp of sequence adjacent to the 20 bp from the plasmid. The adjacent 30 bp did not align to the plasmid contiguously to the known 20 bp. When the 20 bp from the plasmid and the adjacent 30 bp are combined into a 30_20 mer, they indicate the junction shown by the chimeric read. Junction reads were condensed into a unique junction if their 30_20 mers were identical, or if the 30_20 mer junctions were within 2 bp. The total number of sequence reads (referred to as "TotalSupportingReads") for each unique junction was retained for filtering. Junctions with fewer than five unique supporting reads, or if the "TotalSupportingReads" value was below 10% of the median sequencing depth for positions aligned to the plasmid, were filtered and removed from further analysis (Figure 10, Step 8).

Junction Identification

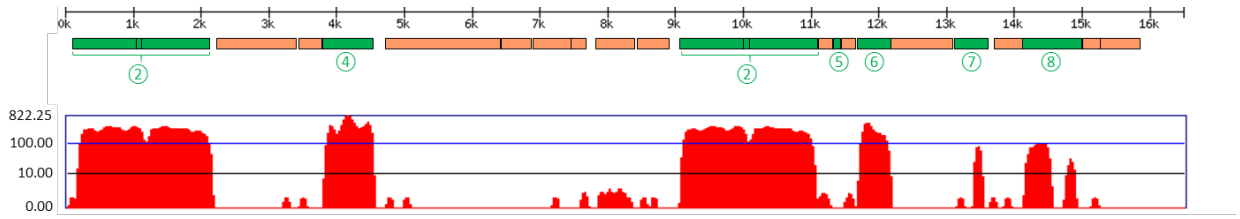
Variations between the maize reference genome used in the SbS analysis and the control maize genome may result in identification of junctions that are due to these differences in the endogenous maize sequences. To detect these endogenous junctions, control maize genomic DNA libraries were captured and sequenced in the same manner. These libraries were sequenced to an average depth approximately five times that of the depth for the DP23211 maize plant samples. This increased the probability that the endogenous junctions captured by the plasmid probes would be detected in the control maize samples, so that they could be identified and removed from the DP23211 maize samples. The 30_20 mers of the endogenous junctions detected in this analysis were used to filter the same endogenous junctions in the DP23211 maize samples (Figure 10, Step 8), so that the only junctions remaining in the DP23211 samples are due to actual insertions derived from PHP74643, PHP56614, PHP21139, or PHP31729 (Figure 10, Step 9).

SbS Results

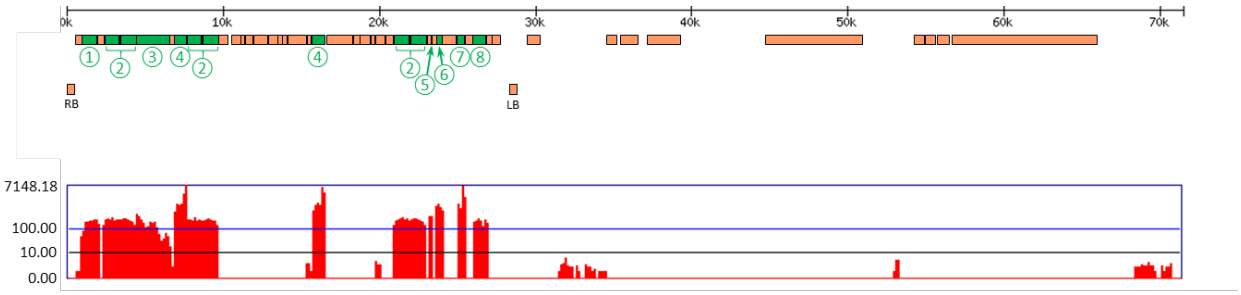
Results for the control maize, positive control, and DP23211 maize "representative plant" (Plant ID 343210845) are presented in the main body (V-B. Southern-by-Sequencing (SbS) Analysis for Copy Number, Integrity, and Confirmation of the Absence of Vector Backbone Sequence) of this document.

Remaining plant results from SbS analysis are presented in Figure 44, Figure 45, Figure 46, Figure 47, Figure 48, Figure 49, Figure 50, Figure 51, and Figure 52 below:

A. Alignment to Intended Insertion

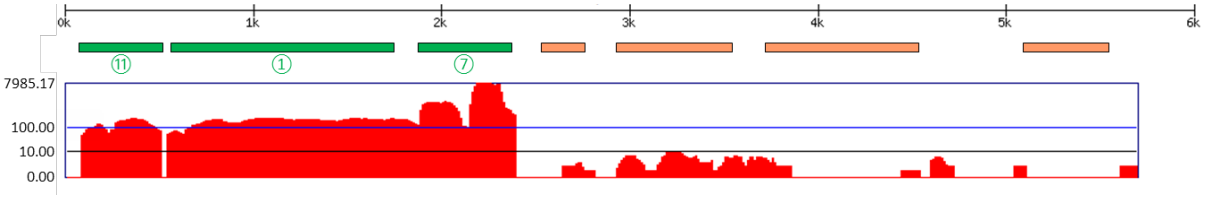


B. Alignment to PHP74643



C. Alignment to PHP56614

D. Alignment to PHP21139



E. Alignment to PHP31729

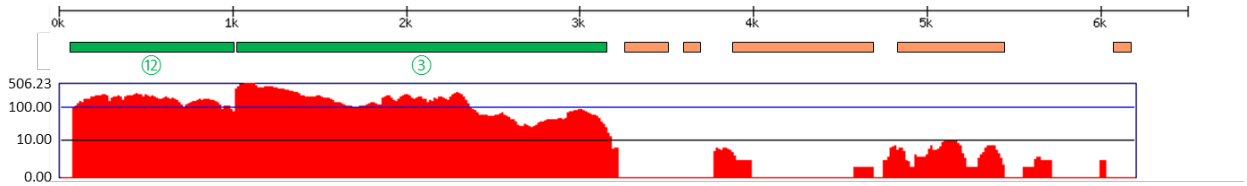
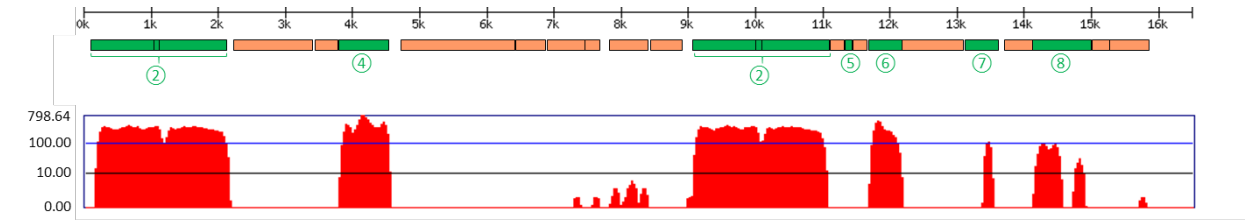


Figure 44. SbS Results for DP23211 Maize (Plant ID 343210846)

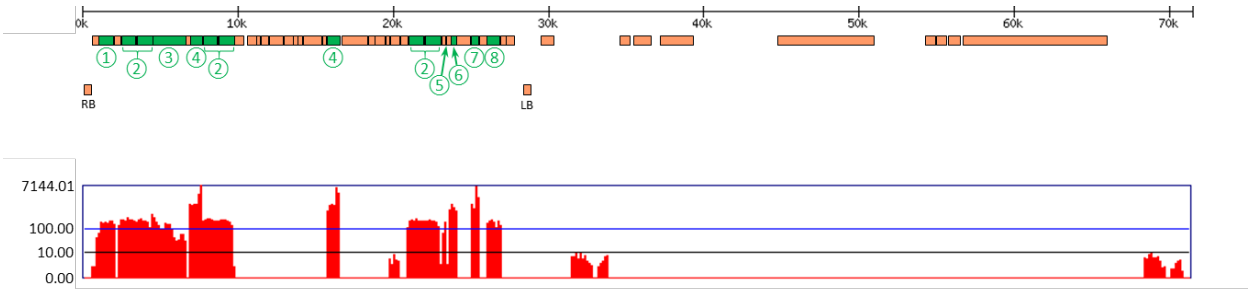
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other

sources. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant does not contains the intended insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequence reads are solely due to the endogenous elements present in the maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or backbone sequence present in this plant from the T1 generation of DP23211 maize.

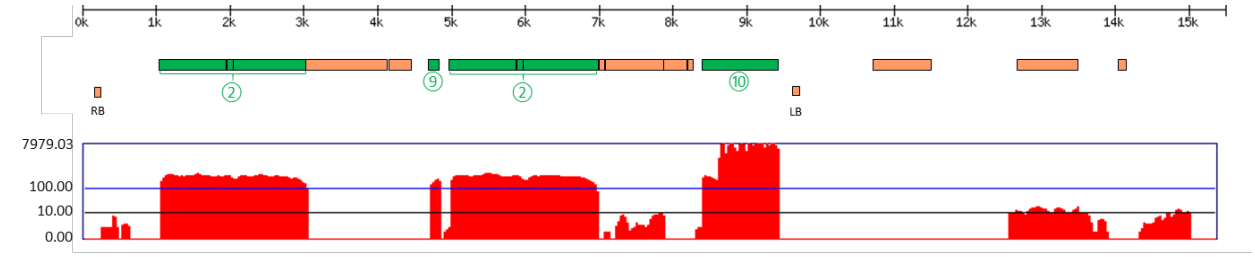
A. Alignment to Intended Insertion



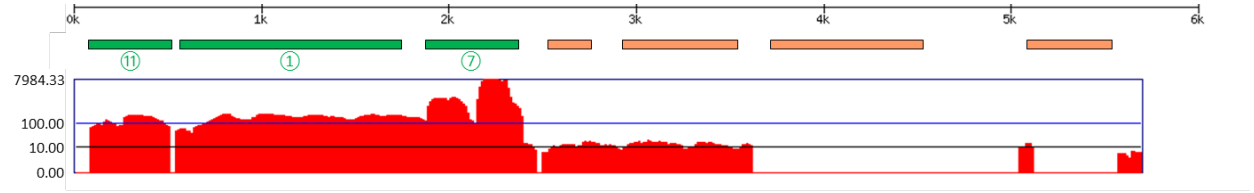
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

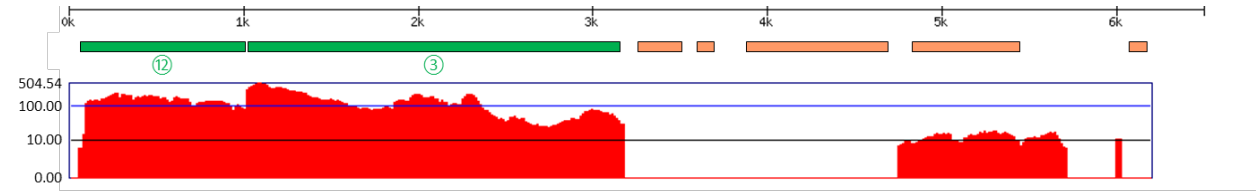
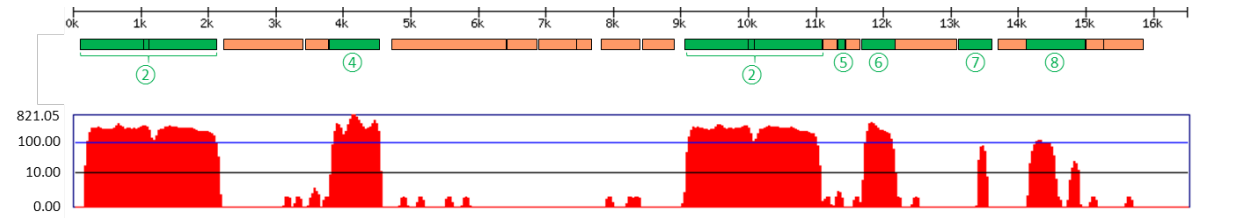


Figure 45. SbS Results for DP23211 Maize (Plant ID 343210847)

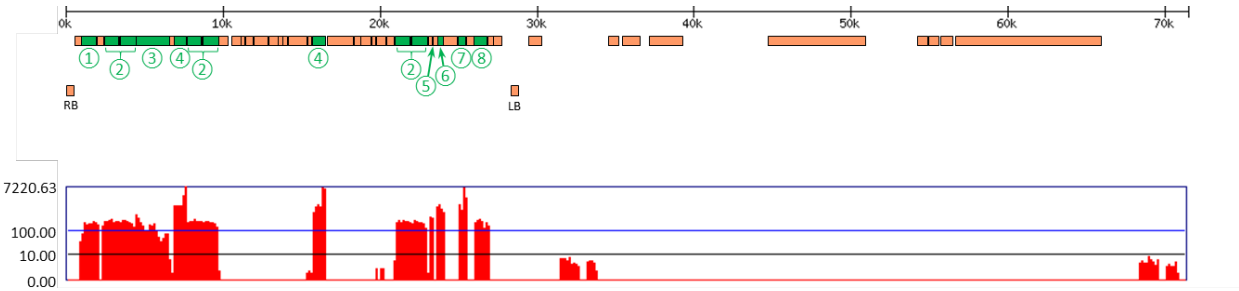
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other

sources. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant does not contains the intended insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequence reads are solely due to the endogenous elements present in the maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or backbone sequence present in this plant from the T1 generation of DP23211 maize.

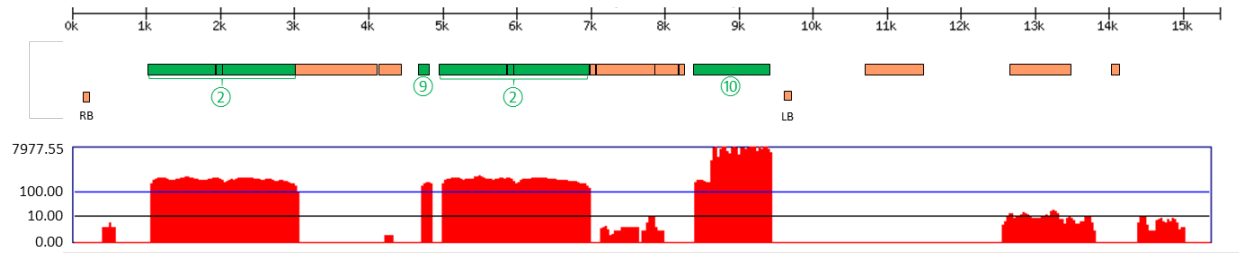
A. Alignment to Intended Insertion



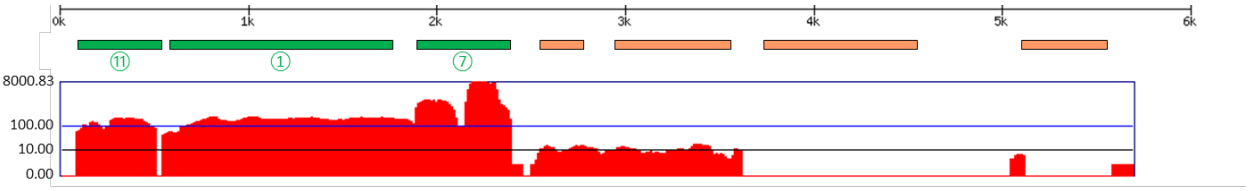
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

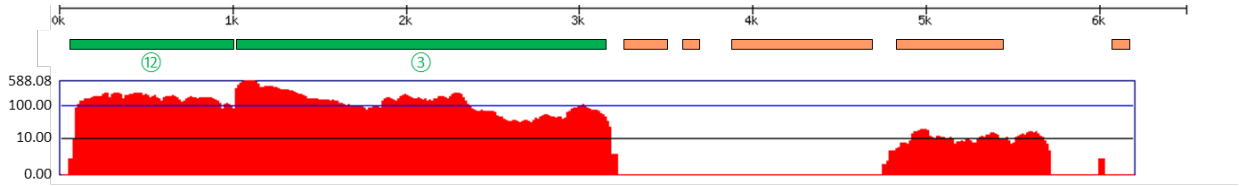
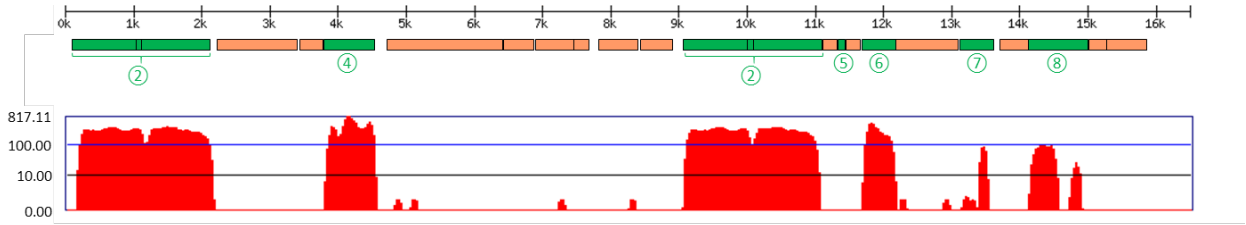


Figure 46. SbS Results for DP23211 Maize (Plant ID 343210848)

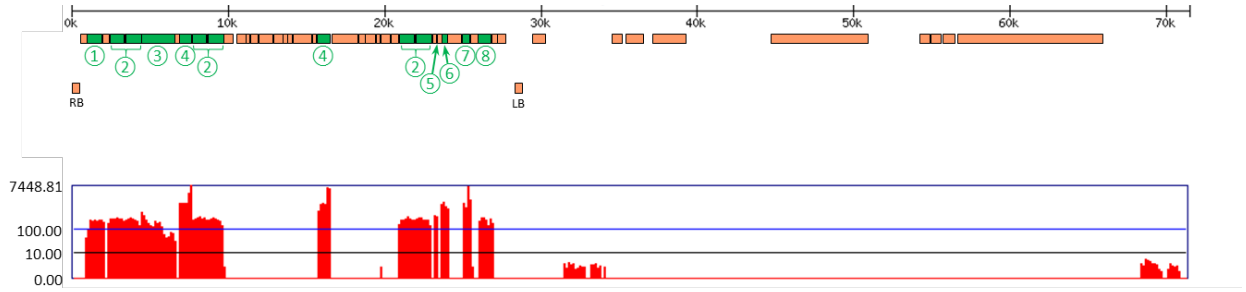
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other

sources. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant does not contains the intended insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequence reads are solely due to the endogenous elements present in the maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or backbone sequence present in this plant from the T1 generation of DP23211 maize.

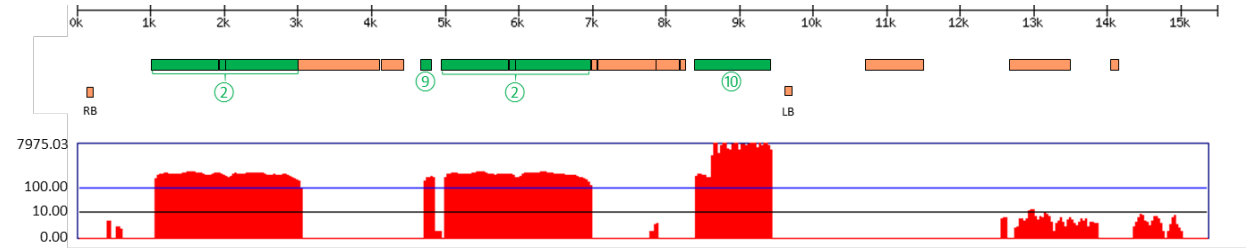
A. Alignment to Intended Insertion



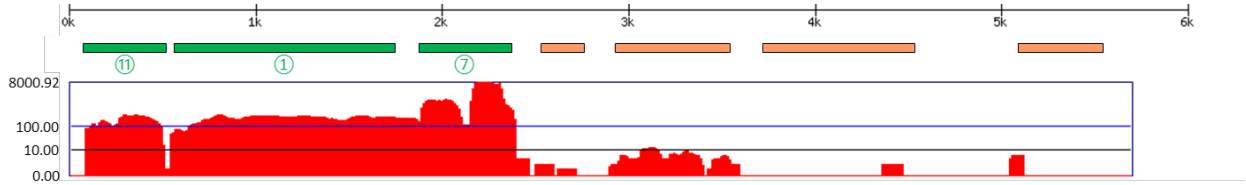
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

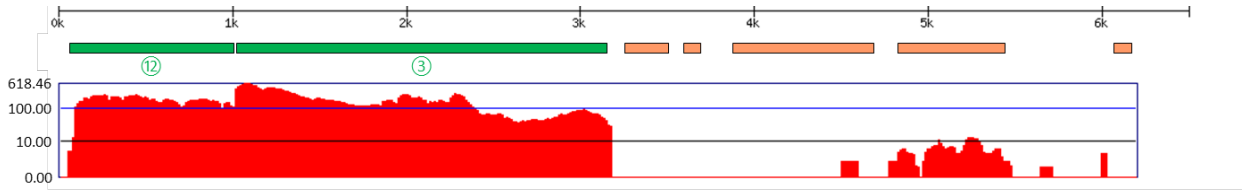
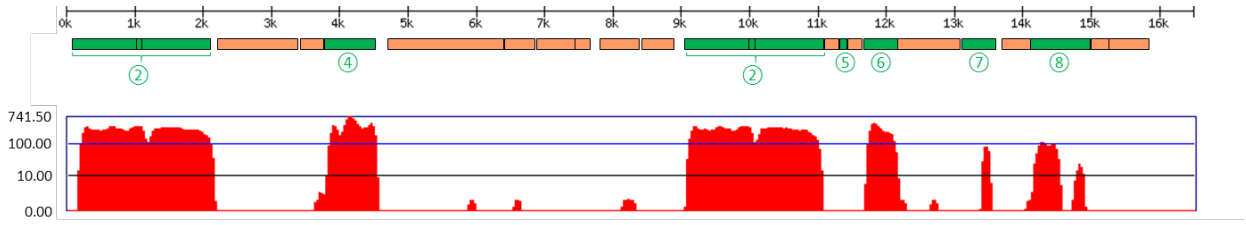


Figure 47. SbS Results for DP23211 Maize (Plant ID 343210849)

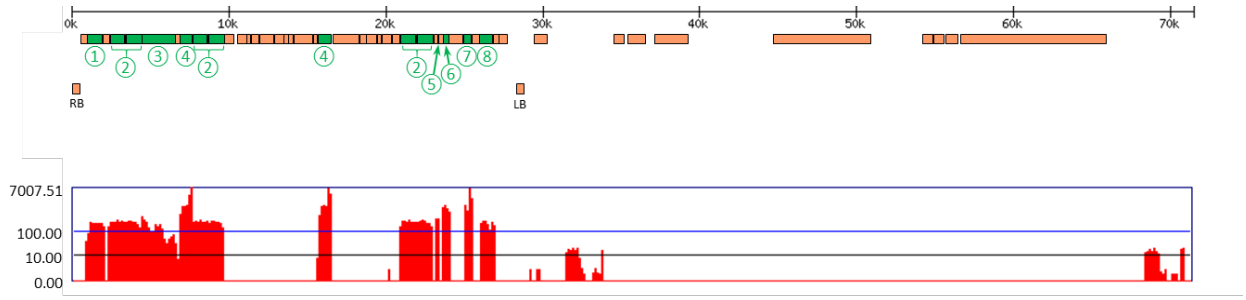
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other

sources. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant does not contains the intended insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequence reads are solely due to the endogenous elements present in the maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or backbone sequence present in this plant from the T1 generation of DP23211 maize.

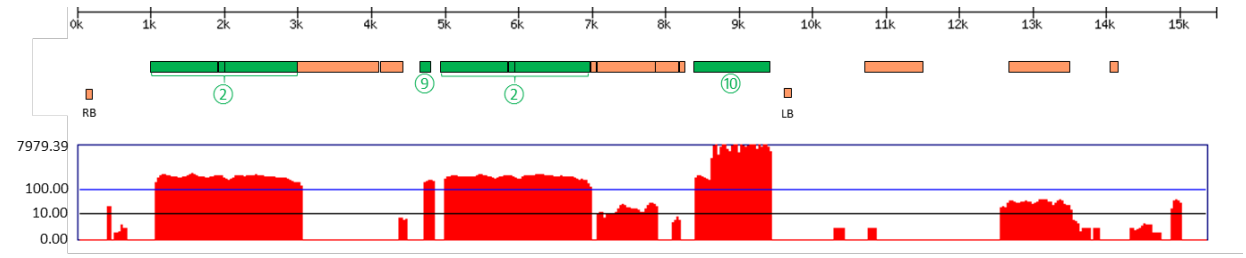
A. Alignment to Intended Insertion



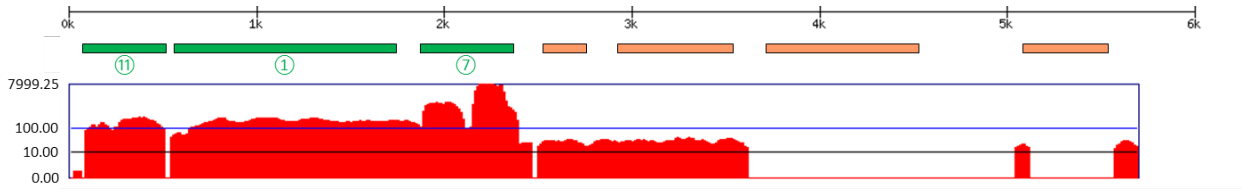
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

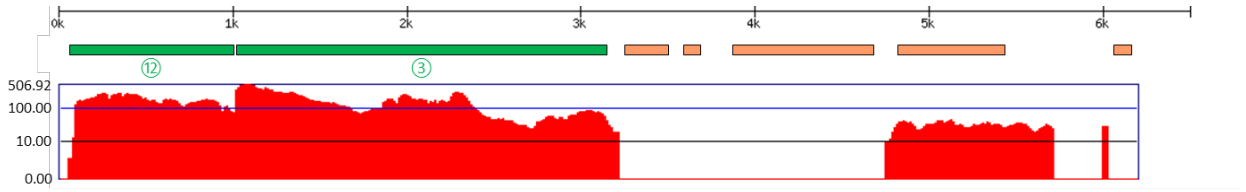
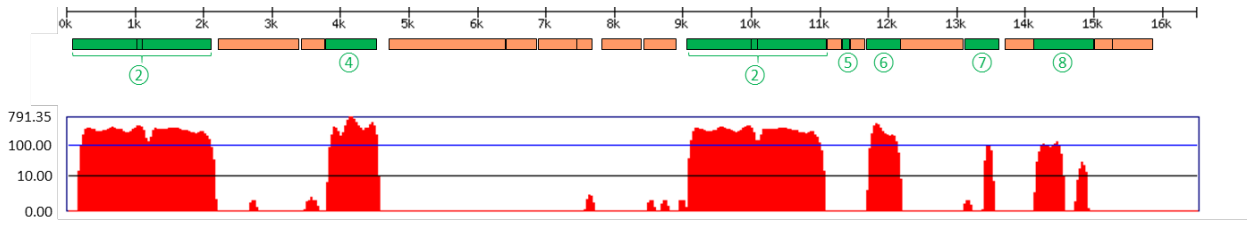


Figure 48. SbS Results for DP23211 Maize (Plant ID 343210850)

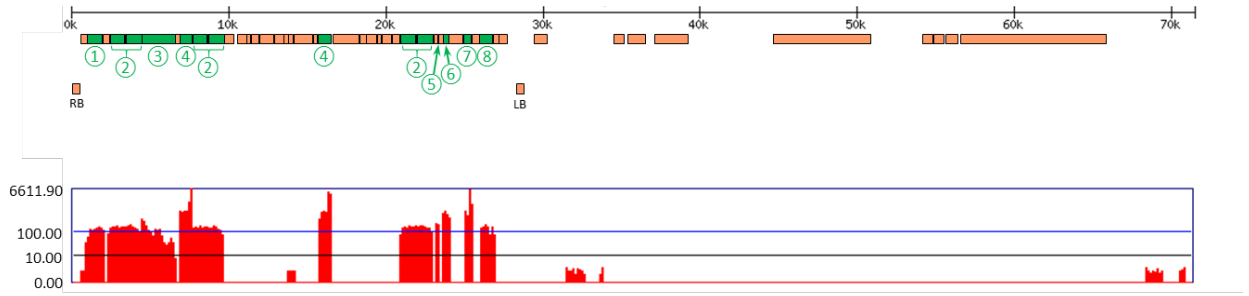
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other

sources. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant does not contains the intended insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequence reads are solely due to the endogenous elements present in the maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or backbone sequence present in this plant from the T1 generation of DP23211 maize.

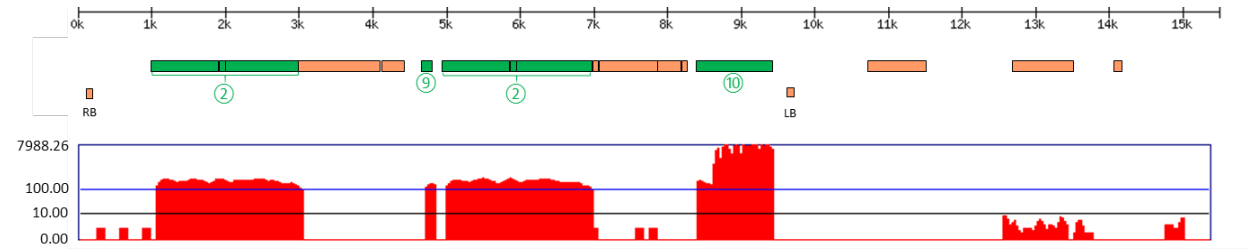
A. Alignment to Intended Insertion



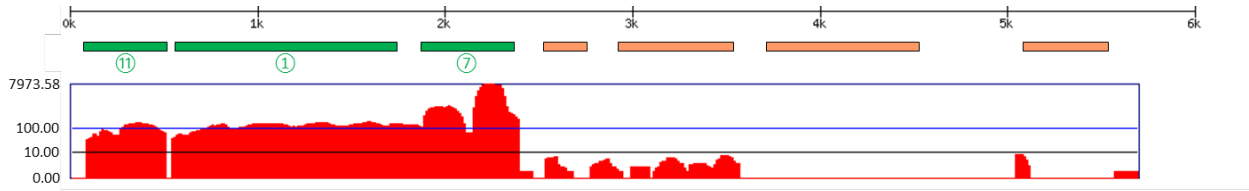
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

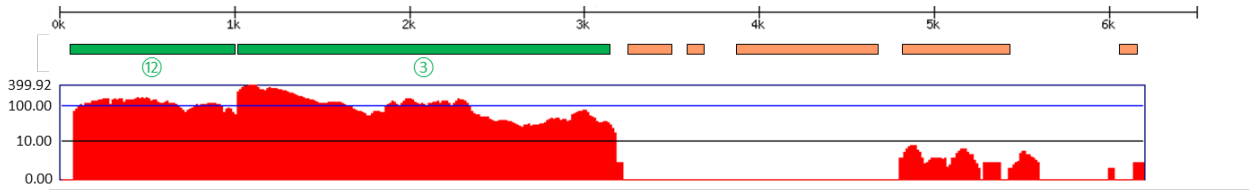
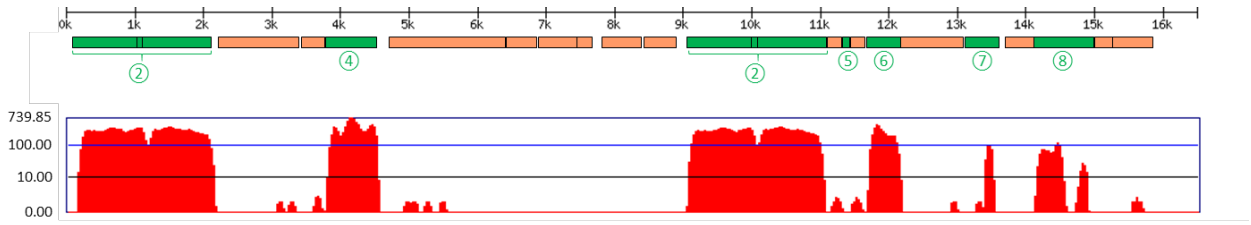


Figure 49. SbS Results for DP23211 Maize (Plant ID 343210851)

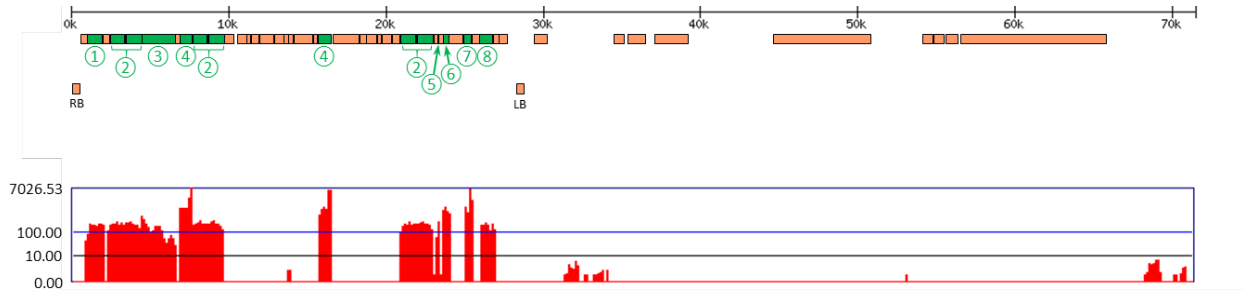
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other

sources. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant does not contains the intended insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequence reads are solely due to the endogenous elements present in the maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or backbone sequence present in this plant from the T1 generation of DP23211 maize.

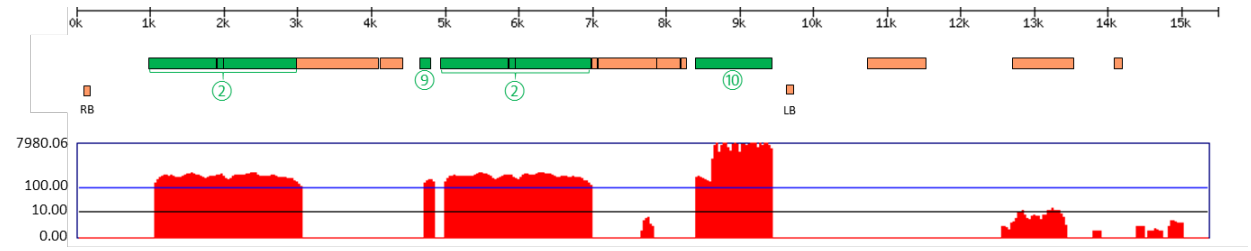
A. Alignment to Intended Insertion



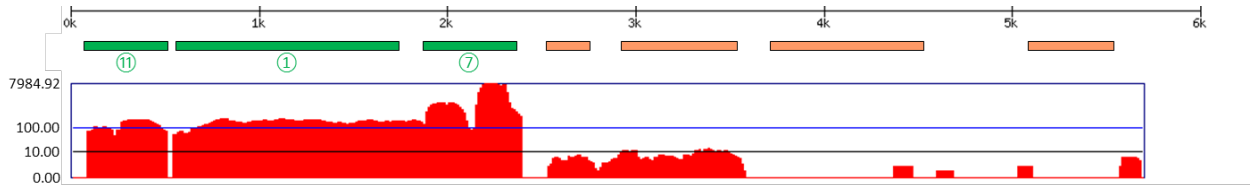
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

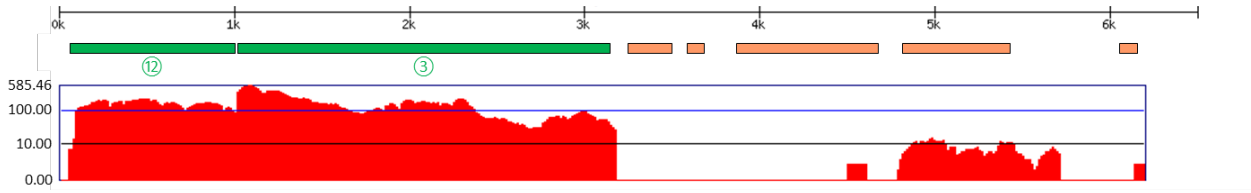
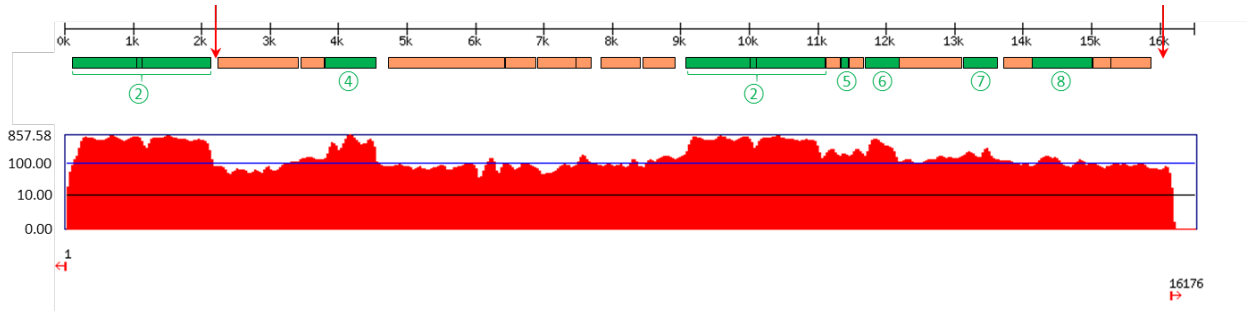


Figure 50. SbS Results for DP23211 Maize (Plant ID 343210852)

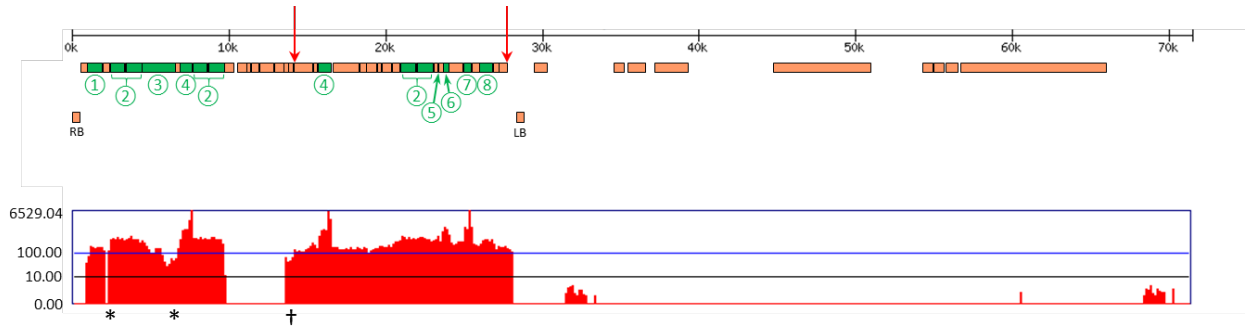
The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other

sources. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant does not contains the intended insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequence reads are solely due to the endogenous elements present in the maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or backbone sequence present in this plant from the T1 generation of DP23211 maize.

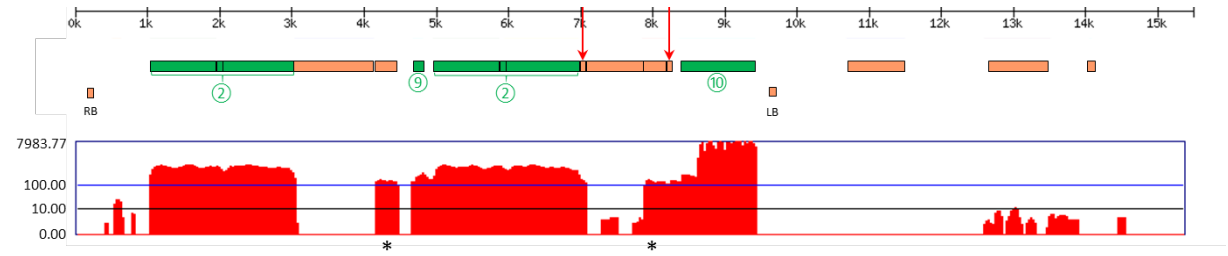
A. Alignment to Intended Insertion



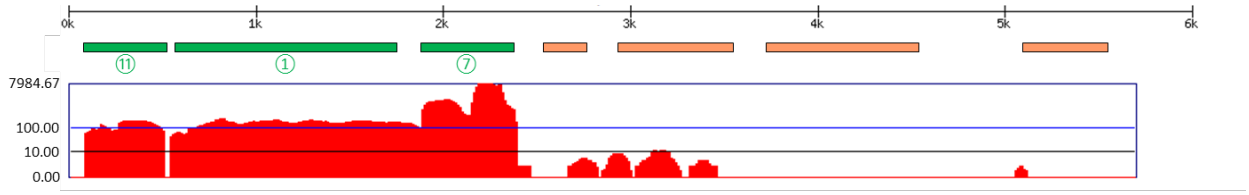
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

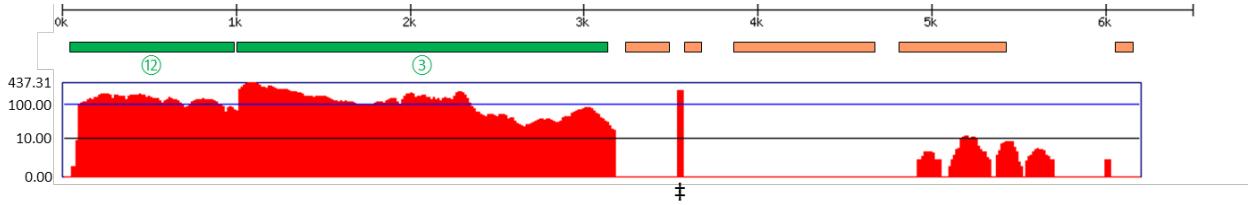
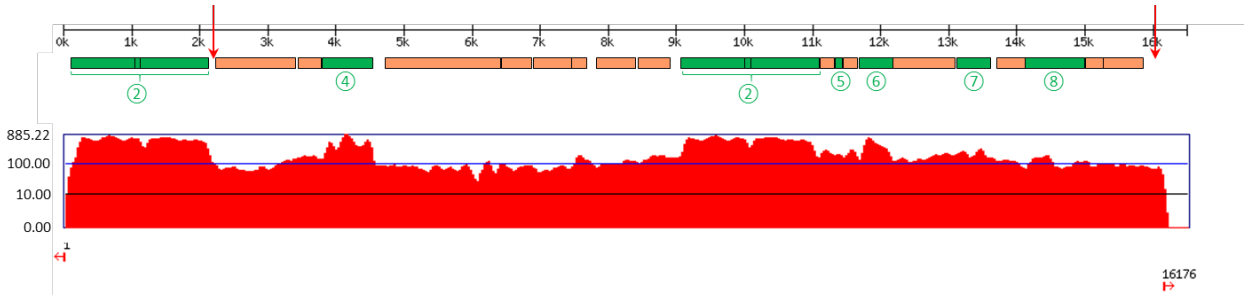


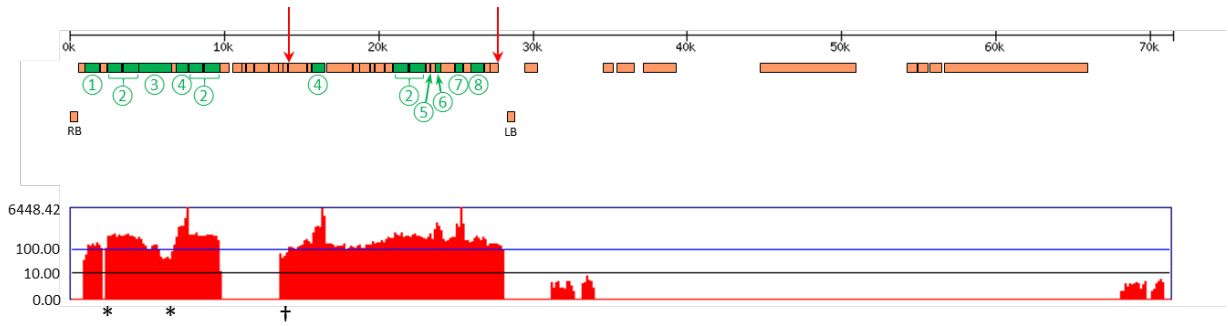
Figure 51. SbS Results for DP23211 Maize (Plant ID 343210853)

The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant contains the intended insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the intended insertion (Figure 7). The presence of only two junctions demonstrates the presence of a single insertion in the DP23211 maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained for the elements between FRT1 and FRT87 transferred into DP23211 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region from approximately 1k to 10k that were not transferred into the DP23211 maize genome, and to the *pin*II terminator (*) and CaMV35S terminator (†) elements outside of the FRT sites due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in PHP74643. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained for *zm*-SEQ9, *zm*-SEQ8, the elements found in the intended insertion (between *zm*-SEQ9 to FRT1 and between FRT87 to *zm*-SEQ8), and for the endogenous elements not in the intended insertion (the *ubi*ZM1 promoter, 5' UTR, and intron in the I-*Cre*I cassette), along with the *pin*II terminator elements (*) in PHP56614 due to alignment of reads derived from the *pin*II terminator in the *pmi* cassette of the intended insertion to the two copies of this element in PHP56614. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained for the endogenous elements and for a small segment of an *att* recombination site that matches an *att* site found in the intended insertion (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or backbone sequence present in DP23211 maize.

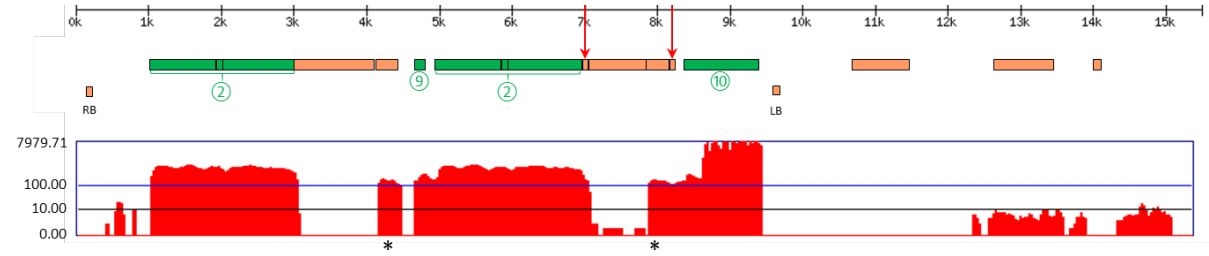
A. Alignment to Intended Insertion



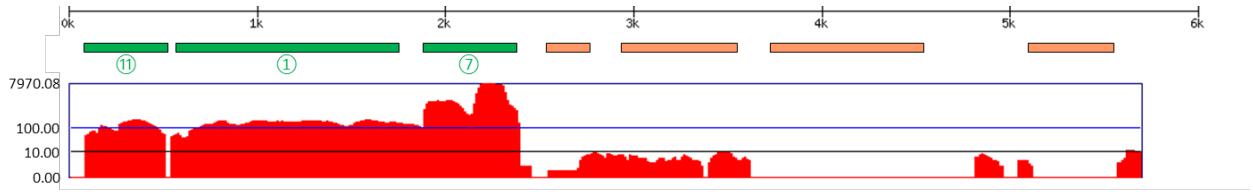
B. Alignment to PHP74643



C. Alignment to PHP56614



D. Alignment to PHP21139



E. Alignment to PHP31729

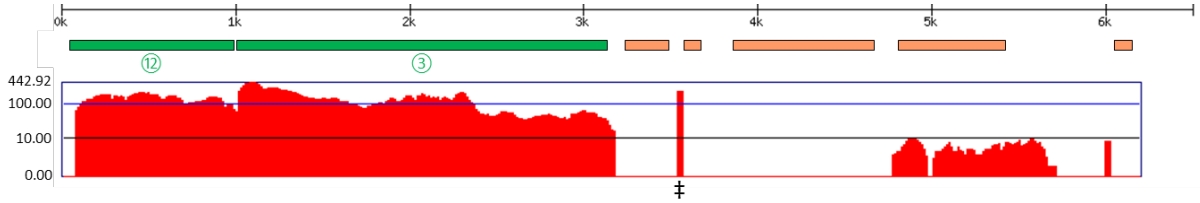


Figure 52. SbS Results for DP23211 Maize (Plant ID 343210854)

The red coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion (16,176 bp; Figure 7), indicating that this plant contains the intended insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the intended insertion (Figure 7). The presence of only two junctions demonstrates the presence of a single insertion in the DP23211 maize genome. **B)** SbS results aligned against the plasmid PHP74643 sequence (71,116 bp; Figure 5). Coverage was obtained for the elements between FRT1 and FRT87 transferred into DP23211 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region from approximately 1k to 10k that were not transferred into the DP23211 maize genome, and to the *pin*II terminator (*) and CaMV35S terminator (†) elements outside of the FRT sites due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in PHP74643. **C)** SbS results aligned against the plasmid PHP56614 sequence (15,339 bp; Figure 1). Coverage was obtained for *zm*-SEQ9, *zm*-SEQ8, the elements found in the intended insertion (between *zm*-SEQ9 to FRT1 and between FRT87 to *zm*-SEQ8), and for the endogenous elements not in the intended insertion (the *ubi*ZM1 promoter, 5' UTR, and intron in the I-*Cre*I cassette), along with the *pin*II terminator elements (*) in PHP56614 due to alignment of reads derived from the *pin*II terminator in the *pmi* cassette of the intended insertion to the two copies of this element in PHP56614. **D)** SbS results aligned against the plasmid PHP21139 sequence (5,687 bp; Figure 3). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid PHP31729 sequence (6,181 bp; Figure 4). Coverage was obtained for the endogenous elements and for a small segment of an *att* recombination site that matches an *att* site found in the intended insertion (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or backbone sequence present in DP23211 maize.

Appendix 3. Materials and Methods for Southern Blot Analysis

Seed from each of the five generations of DP23211 maize and the control maize were planted in a controlled environment at Pioneer, Johnston, Iowa, USA. Fresh leaf tissue samples from test and control maize were harvested and then lyophilized. Lyophilized tissue samples were shipped to Regulatory Sciences, Multi Crop Research Center, Pioneer Hi-Bred Private Limited (Hyderabad, India) at ambient temperature. Upon arrival, samples were stored frozen (< -50°C freezer unit) until processing.

Samples

Genomic DNA was isolated and analyzed from leaf tissue from five generations (one plant from each of the T1, T2, T3, T4, and T5 generations) of DP23211 maize and one plant from the PHR03 non-GE near isoline control maize.

DNA Extraction and Quantification

The leaf samples were pulverized with steel beads in tubes using a paint shaker (AGS Transact Technology Ltd.). Care was taken to ensure leaf samples were ground sufficiently for DNA isolation. Genomic DNA was isolated using a high salt extraction buffer (2.0 M Sodium chloride, 100 mM Tris-Hydrochloride pH-8.0, 50 mM Sodium salt of EDTA, 3% β-mercaptoethanol (v/v) and 100 mM Sodium metabisulphite) and sequentially precipitated using potassium acetate and isopropyl alcohol. DNA was treated with Ribonuclease A, purified and precipitated using sodium acetate and chilled ethanol. Following the extraction, DNA was quantified using PicoGreen® reagent (Molecular Probes, Invitrogen) and visualized on a 1% agarose gel to check the quality of the isolated DNA.

Digestion of DNA and Electrophoretic Separation

Genomic DNA isolated from both test and control maize leaves was digested with the restriction enzyme *Kpn* I (Thermo Fisher Scientific.). PHP74643 plasmid DNA was added to the control maize DNA samples at a level equivalent to one plasmid copy per genomic copy and digested in the same manner. Following digestion with the restriction enzyme, the fragments produced were electrophoretically separated according to their sizes using an agarose gel and documented by photographing the gel under UV illumination (BioRad Gel doc XR+ System.).

Southern Transfer

The DNA fragments separated on the agarose gel were denatured *in situ*, transferred to a nylon membrane (GE Healthcare, LC) and fixed to the membrane by UV crosslinking (UV Stratalinker, UVP).

Probe Labeling and Southern Blot Hybridization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probe. DNA probes specific to the *pmi, mo-pat* and *ipd072Aa* gene and DvSSJ1 fragment elements were labeled by incorporation of Digoxigenin (DIG) labeled nucleotide (DIG-11-dUTP) into the fragments. Labeled probes were hybridized to the DNA on the nylon membrane for detection of the specific genomic DNA fragments. DNA Molecular Weight Marker III and VII, Digoxigenin (DIG) labeled (Roche) were used for visualization as the fragment size standards on the blot.

Detection of Hybridized Probes

After stringent washes, DIG-labeled DNA standards and single stranded DIG-labeled probes hybridized to DNA bound to the nylon membrane were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed for one or more time points to detect hybridizing fragments and to visualize molecular weight standards. Images were captured by detection with the Syngene G-Box Chemi XT16 and XX6 (Syngene, Inc.). Detected bands were documented for each probe.

Stripping of Probes and Subsequent Hybridization

Following hybridization and detection, membranes were stripped of DIG-labeled probe to prepare blot for subsequent re-hybridization to a different probe. Membranes were rinsed briefly in distilled and de-ionized water and then stripped in a solution of 0.2N NaOH and 0.1% SDS at 37°C with constant shaking. The membranes were then rinsed in 2x SSC and either used directly for subsequent hybridizations or stored for later use. The alkali based stripping procedure effectively removed probes labeled with alkali-labile DIG used in these experiments.

Appendix 4. Materials and Methods for Segregation Analysis of Five Generations of DP23211 Maize

Genotypic and phenotypic analyses were conducted for five generations of DP23211 maize (BC1F1 in genetic background PH1V5T, BC2F1, T1, T5, and BC1F1 in genetic background PH2SRH). The genotypic analysis evaluated each individual plant for the presence or absence of the following genes or genetic elements by qualitative polymerase chain reaction (qPCR): event DP-Ø23211-2, *ipd072, mo-pat, pmi,* and *DvSSJ1.* The genotypic analysis also evaluated each individual plant for the presence or absence of the following genes or genetic elements by endpoint PCR: *STOPS2-UBI1, AT-T9-STOPS3, ATTB2-S2-BSV*. The phenotypic analysis evaluated tolerance to glufosinate for each individual plant. The individual results for each plant were compared to the qPCR results to verify co-segregation of genotype with phenotype.

Bias in this study was controlled through uniform maintenance of all entries during the entire study period.

Planting and Thinning

For the BC1F1 in genetic background PH1V5T, BC2F1, T1, T5, and BC1F1 in genetic background PH2SRH generations of DP23211 maize, more than 100 seeds were planted. Seeds were planted in separate 4-inch pots (one seed per pot, organized in flats containing 15 pots) and grown in a controlled environment under conditions for producing maize plants. Fourteen to seventeen days after planting, plants were thinned by removing unhealthy plants. The generation of DP23211 maize was thinned to the following final populations: BC1F1 in genetic background PH1V5T, BC2F1, T5, T1, and BC1F1 in genetic background PH2SRH generations of DP23211 maize were thinned to at least 100 plants.

PCR Analysis

PCR Sample Collection

For all generations intended for genotypic and phenotypic analysis (BC1F1 in genetic background PH1V5T, BC2F1, T1, T5, and BC1F1 in genetic background PH2SRH), one sample per plant was collected at the V3 growth stage. Each sample consisted of three leaf punches collected into one bullet tube and placed on dry ice until transferred to a freezer for frozen storage. Individual plants and corresponding leaf samples were uniquely labeled to allow a given sample to be tracked back to the originating plant.

Genotype Analysis

Leaf samples were analyzed using a qPCR assay to confirm the presence or absence of the event DP-Ø23211-2 and to confirm the presence or absence of the genes or genetic elements *ipd072Aa*,

mo-pat, pmi, DvSSJ1, and event DP-Ø23211-2. Leaf samples were also analyzed using endpoint PCR for the following genes or genetic elements; *STOPS2-UBI1, AT-T9-STOPS3,* and *ATTB2-S2-BSV*.

Phenotype Analysis

At the V4 to V5 growth stage, glufosinate was applied to each DP23211 maize generation. The spray mixture consisted of Ignite 280 SL containing 24.5% glufosinate-ammonium and ammonium sulfate at a rate of approximately 3.0 lb/A (3.4 kg/ha). No other adjuvants or additives were included in the spray mixture. Ignite 280 SL was applied at a target rate of 22 fl oz/A (1.66 L/ha) with a total spray volume of approximately 33 gal/A (312.4 L/ha) using a spray chamber to simulate a broadcast (over-the-top) application. Actual application rates were within 90-110% of the target herbicide application rate.

Four to five days after herbicide application, each plant was visually evaluated for herbicide tolerance in which presence of herbicide injury corresponded to an herbicide-susceptible phenotype and absence of herbicide injury corresponded to an herbicide-tolerant phenotype.

Correlation of Genotypic and Phenotypic Results

Genotypic and phenotypic results for each plant were compared to verify that PCR assay results and herbicide tolerance were in correlation.

Statistical Analysis

A chi-square test was performed at the 0.05 significance level on the segregation results of each DP23211 maize generation. All plants were identified as either positive or negative, where positive plants were tolerant to glufosinate and confirmed to contain all the transgenes and negative plants were susceptible to glufosinate and did not containing the transgenes. All plants were identified as one of these two categories. A chi-square test was performed separately for each generation to compare the observed segregation ratio to the expected segregation ratio (1:1 for BC1F1 in genetic background PH1V5T, BC2F1, BC1F1 in genetic background PH2SRH, and T1). Chi-square test was not performed for the T5 generation because all plants were identified as positive (i.e., not segregating) as expected for a homozygous generation. Statistical analyses were conducted using SAS software, Version 9.4.

Appendix 5. Materials and Methods for Characterization of IPD072Aa, PAT, and PMI Proteins

Materials and Methods for Characterization of IPD072Aa Protein from DP23211 Maize

Test Substance

The test substance consisted of IPD072Aa protein isolated from tissue derived from DP23211 maize. The whole plant tissue was collected at the V9 growth stage (the stage when the collar of the ninth leaf becomes visible; Abendroth et al., 2011) of development from plants grown at a Pioneer-owned field location (Johnston, IA, USA). The tissue was lyophilized, homogenized and stored at ≤ -50 °C.

Protein Extraction, Purification, and Concentration

The IPD072Aa protein was extracted from lyophilized maize tissue by homogenization with a Waring blender using chilled phosphate-buffered saline containing polysorbate 20 (PBST) extraction buffer (25 ml buffer per g tissue). The sample extract was then filtered through cheesecloth and clarified by centrifugation. Ammonium sulfate (AS) precipitation was used to further purify and concentrate the sample extract. Beginning at 0% AS saturation, AS was slowly added to the sample extract while stirring until 60% AS saturation was reached. The sample was centrifuged and the AS process was repeated with the supernatant, this time beginning at 60% AS saturation and progressing to 80%. The sample was centrifuged again and the fractionated pellet was solubilized in phosphate-buffered saline prior to running the sample through a desalting column. The eluted fraction was further purified by immunoaffinity chromatography. The immunoaffinity column was prepared by coupling an IPD072Aa protein mouse monoclonal antibody (21F1.E5) to AminoLink Plus Coupling Gel. Elutions 2-5 from the immunoaffinity purification were concentrated into one sample using a centrifugal concentrator (10K; Sartorius) and buffer exchanged to a volume of approximately 150 µl.

Following extraction, purification, and concentration, the final volume in the concentrator was estimated and 25% 4X NuPAGE LDS and 10% 10X NuPAGE Sample Reducing Agent was added to the concentrated sample. The sample in the concentrator was heated for 2-5 minutes at 70-100 °C and then transferred to a microcentrifuge tube. The sample was then heat treated at 90-100 °C for 5 (±1) minutes and stored frozen at ≤ -10 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The LDS-treated sample stored at ≤ -10 °C was re-heated for 5 minutes at 90-100 °C and then loaded into 4-12% Bis-Tris gels. Prestained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into each gel to provide a visual verification that migration was within the range of the predicted molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES SDS running buffer and NuPAGE Antioxidant at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for N-terminal amino acid sequencing and peptide mapping.

For Coomassie staining, the gel was washed with ultrapure (American Society for Testing and Materials (ASTM) Type 1) water (referred to as water) 3 times for 5 minutes each and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, the gel was de-stained with water 4 times for at least 5 minutes each until the gel background was clear. Proteins were stained as blue-colored bands on the gel. The gel image was captured electronically using an imaging system (Bio-Rad ChemiDoc MP).

Western Blot Analysis

Following SDS-PAGE as described above, the resulting gel was assembled into a nitrocellulose (NC) iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gel to the NC membrane for 7 minutes with a pre-set program (P3).

Following protein transfer, the membrane was blocked in PBST containing 5% weight/volume (w/v) non-fat dry milk for 60 minutes at ambient laboratory temperature. Before and after the blocking step, the membrane was washed with PBST 3 times for 1 minute each to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk. Following primary antibody incubation, the membrane was washed 4 times in PBST for 5 minutes each. The membrane was incubated for 60 minutes at ambient laboratory temperature with a secondary antibody (anti-rabbit IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% non-fat dry milk. The membrane was washed 4 times with PBST for 5 minutes each. The blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

Peptide Mapping and Sequencing Analysis by LC-MS Analysis

Following SDS-PAGE, Coomassie staining, and gel imaging using the methods as described above, two IPD072Aa protein bands were excised from a gel and stored frozen at ≤ -10 °C. The protein in each gel slice was reduced with DTT, alkylated with iodoacetamide, and then subsequently digested with trypsin or chymotrypsin. The digested samples were separated on an ACQUITY UPLC (Waters Corporation) fitted with a Cortecs UPLC C18 1.6 µm Column (2.1 x 100 mm) (Waters Corporation) by gradient elution. Eluent from the column was directed into an electrospray source, operating in positive mode, on a TripleTOF 5600+ hybrid quadrupole-TOF mass spectrometer (AB Sciex). The resulting MS data were processed using MS Data Converter (Beta 1.3) to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software version 2.6.1) and match peptides from the expected IPD072Aa protein sequence (Perkins et al., 1999). The following search parameters were used: peptide and fragment mass tolerance, ± 0.1 Da; fixed modifications, cysteine carbamidomethyl; variable modifications, methionine oxidation; maximum missed cleavages, 1 for trypsin and 2 for chymotrypsin. The Mascot-generated peptide ion score threshold was >13 which indicates identity or extensive homology ($p<0.05$). The combined sequence coverage was calculated with GPMAW version 9.2.

N-Terminal Amino Acid Sequence Analysis

Following SDS-PAGE as described above, the resulting gel was incubated in cathode buffer (60 mM Tris, 40 mM CAPS, 0.075% SDS, pH 9.6) for 10-20 minutes. An Immobilon-P PVDF membrane was wetted in 100% methanol for 1 minute, followed by immersion in anode buffer (60 mM Tris, 40 mM CAPS, 15% methanol, pH 9.6) for 10-15 minutes. A Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system was used to transfer proteins from the gel to the membrane at 12 V for 45 minutes. Following protein transfer, the membrane was then stained with GelCode Blue stain reagent for 10 minutes and then destained with water and 50% methanol to visualize the IPD072Aa protein band. Two bands containing the maize-derived IPD072Aa protein were excised and stored frozen at ≤ -10 °C. Both bands were analyzed as a single sample using a Shimadzu PPSQ-51A sequencer. Ten cycles of Edman sequencing were performed. During each cycle, the N-terminal amino acid was sequentially derivatized with phenylisothiocyanate (PITC), cleaved with trifluoracetic acid, and converted to PTH-amino acid which was identified through chromatography. LabSolutions Software was used to automatically identify the N-terminal sequence.

Protein Glycosylation Analysis

The Pierce Glycoprotein Staining Kit was used to determine whether the IPD072Aa protein was glycosylated. The IPD072Aa protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor), were run by SDS-PAGE as described above.

Following electrophoresis, the gel was washed with water twice for 5 minutes each wash, fixed with 50% methanol for 30-35 minutes, and washed twice with 3% acetic acid for 10-15 minutes each wash. The gel was then incubated with oxidizing solution for 15-20 minutes and washed three times with 3% acetic acid for 5-7 minutes each wash. The gel was incubated with glycoprotein staining reagent for 15-20 minutes and then incubated in a reducing reagent for 5-7 minutes. The gel was then washed with 3% acetic acid for 5 minutes and then rinsed in water for 5 minutes. Glycoproteins were detected as magenta colored bands on the gel.

Following glycoprotein detection, the image of the gel was captured electronically. The same gel was then stained with GelCode Blue stain reagent for 60 minutes followed by 3 washes with water (at least 5 minutes each) to visualize all protein bands. The image of the GelCode stained gel was then captured electronically.

Biological Activity of Heat-Treated IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder which was produced from recombinant bacteria (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under the stated storage condition.

Carrier

The carrier consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2-7 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet. The dosing solutions used to prepare Treatments 3-6 were incubated for 30-35 minutes at various temperatures. The dosing solution used to prepare Treatment 7 was autoclaved (121 °C, 20 psi) for approximately 30 minutes.

Test System

The test system was WCR. The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

WCR larvae were exposed via oral ingestion to one of the following seven treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (containing an unheated IPD072Aa protein dosing solution)
- Treatment 3: Test Diet (containing an IPD072Aa protein dosing solution incubated at 25 °C)
- Treatment 4: Test Diet (containing an IPD072Aa protein dosing solution incubated at 50 °C)
- Treatment 5: Test Diet (containing an IPD072Aa protein dosing solution incubated at 60 °C)
- Treatment 6: Test Diet (containing an IPD072Aa protein dosing solution incubated at 95 °C)
- Treatment 7: Test Diet (containing an IPD072Aa protein dosing solution autoclaved at 121 °C)

Each test diet contained a targeted concentration of 50 ng IPD072Aa protein per mg diet wet weight. Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3 replicates from each treatment. Each treatment was provided to a target of 30 WCR individuals.

When we refer to a generalized randomized block design, we follow the definition given by Kutner et al. (2005). That is, a design with multiple replicates/experimental units assigned to each treatment within each block. This design is sometimes referred to as a general complete block design. Some authors do not differentiate generalized randomized block designs from blocking designs with a single replicate of each treatment per block. In this study, each block consisted of a 24-well bioassay plate and contained 3 replicates (individual insects) receiving each treatment.

The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 4. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment 1) group.

Western blot analysis was used to visually confirm the dose, homogeneity, and stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatment 1 and stability of the IPD072Aa protein test dosing solution used to prepare Treatment 2 were also assessed.

Bias in the WCR bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation

A bulk bioassay control dosing solution was prepared. In addition, a bulk test dosing solution was prepared and aliquots were incubated. On each day of diet preparation, the bioassay control dosing solution and test dosing solution aliquots were mixed with carrier in a 2.51:1 ratio (*i.e.*, 2.51 ml of dosing solution to 1 g of carrier), generating Treatments 1-7. Dosing solutions were maintained chilled (in a refrigerator set at 4 °C or on wet ice) until use.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1 and 2 were collected for characterization of diets.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (*i.e.*, 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variable of interest was mortality. Weight data were not statistically compared due to high mortality. Statistical comparisons were made between WCR provided diet containing IPD072Aa protein (Treatments 3-7) and the unheated test diet (Treatment 2) for mortality.

Mortality

Statistical analysis was conducted using Fisher's exact test to compare the mortality rate of WCR provided the artificial insect diet containing IPD072Aa protein (m_T) and the mortality rate of those provided the unheated test diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T = m_c \quad vs. \quad H_a : m_T \neq m_c \ .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test. Fisher's exact test is described in Agresti (2013) among other sources and was first proposed by R. A. Fisher (1935). The test is an appropriate method to compare binomial proportions between pairs of treatments, since the binary outcome and each pair of treatments forms a 2 × 2 contingency table that can be tested for independence. That is, that the probability of mortality is independent of treatment.

Fisher's exact test considers each insect to be a separate experimental unit and thus correctly accounts for the total number of insects tested. A logistic regression analysis could be applied to this type of data. However, logistic regression uses asymptotic theory to conduct inference tests. That is, the hypothesis test P-values are approximations that are exact only as sample sizes approach infinity. An advantage of logistic regression is that it allows for multiple experimental factors to be included in a statistical model. However, diet was the only treatment factor included in this study, making Fisher's exact test a better choice, since it avoids asymptotic theory.

Bioinformatics Assessment of IPD072Aa Protein to Known Allergens

Methods

Two separate searches for the IPD072Aa protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2019 database (January 2019) available at http://comparedatabase.org. This peer-reviewed database is a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins, and Bioinformatics (PATB) Committee and is comprised of 2,081 sequences. The first search used the IPD072Aa protein sequence as the query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the allergen sequences. The search was conducted using default parameters, except the E-score threshold was set to 10-4. An E-score threshold of 10-4 has been shown to be an appropriate value for allergenicity searches (Mirsky et al., 2013). The generated alignments were examined to identify any that are a length of 80 or greater and possess a sequence identity of ≥ 35%. The second search used an in-house Perl script (runLinearEpitopeScreen.pl) to identify any

contiguous 8-residue identical matches between the IPD072Aa protein sequence and the allergen sequences. A summary of the FASTA search parameters is provided in Table 137.

Results and Discussion

Results of the search of the IPD072Aa protein sequence against the COMPARE database of known and putative allergen sequences found no alignments that were a length of 80 or greater with a sequence identity of ≥ 35% (Figure 53). No contiguous 8-residue matches between the IPD072Aa protein sequence and the allergen sequences were identified in the second search (Figure 54). Taken together, the comparisons of the IPD072Aa protein sequence to the allergen sequences showed that there is no apparent allergenicity concern regarding the IPD072Aa protein.

Conclusion

Results of the search of the IPD072Aa protein sequence against the COMPARE database of known and putative allergen sequences found no alignments that were a length of 80 or greater with a sequence identity of ≥ 35%. No contiguous 8-residue matches between the IPD072Aa protein sequence and the allergen sequences were identified in the second search. Taken together, the comparisons of the IPD072Aa protein sequence to the allergen sequences showed that there is no apparent allergenicity concern regarding the IPD072Aa protein.

Table 139. Summary of Database and FASTA Parameters

FASTA Version	Database	Gap Open Penalty	Gap Extension Penalty	*E*-Score Cutoff	Matrix
35.4.4	COMPARE (January 2019)	-10 (Default)	-2 (Default)	10^{-4}	BLOSUM50 (Default)

```
  1 MGITVTNNSS NPIEVAINHW GSDGDTSFFS VGNGKQETWD RSDSRGFVLS
 51 LKKNGAQHPY YVQASSKIEV DNNAVKDQGR LIEPLS
```

Figure 53. Amino Acid Sequence of the IPD072Aa Protein

```
# fasta35 -q -L -H -E 0.0001 IPD072Aa.txt COMPARE2019.txt

FASTA searches a protein or DNA sequence data bank

 version 35.04 Jan. 15, 2009

Please cite:

 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448


Query: IPD072Aa.txt

  1>>>IPD072aa - 86 aa

Library: COMPARE2019.txt  494256 residues in  2081 sequences


 494256 residues in  2081 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 6.0095+/-0.00291; mu= -1.4217+/- 0.151

 mean_var=62.6049+/-15.243, 0's: 1 Z-trim: 5  B-trim: 0 in 0/46

 Lambda= 0.162095

Algorithm: FASTA (3.5 Sept 2006) (optimized)

Parameters: BL50 matrix (15:-5) ktup: 2

 join: 36, opt: 24, open/ext: -10/-2, width:  16

 Scan time:  0.000

!! No sequences with E() < 0.000100



86 residues in 1 query   sequences

494256 residues in 2081 library sequences

 Scomplib (35.04)

 start: Mon Jan 21 08:13:50 2019 done: Mon Jan 21 08:13:50 2019

 Total Scan time:  0.000 Total Display time:  0.000


Function used was FASTA (version 35.04 Jan. 15, 2009)
```

Figure 54. Output from the FASTA Search Between the IPD072Aa Protein Sequence and COMPARE Sequences

```
Potential Linear Epitope Analysis

Query File = IPD072Aa.txt, Library = COMPARE2019.txt

Peptide Cutoff = 8 residues

Results for query IPD072aa:


There were no common 8 residue peptides.


Final Summary:


There were no common 8 residue peptides.
```

Figure 55. Output from the Contiguous 8-Residue Exact Match Search Between IPD072Aa Protein Sequence and COMPARE Sequences

Bioinformatics Assessment of IPD072Aa Protein to Known Toxins

Methods

The comparison of the IPD072Aa protein sequence to the protein sequences in the DuPont Pioneer toxin database (January 10, 2019) was conducted with BLASTP using default parameters, except that low complexity filtering was turned off, the *E*-value threshold was set to 10^{-4}, and unlimited alignments were returned. Any alignment between the IPD072Aa protein and a protein in the DuPont Pioneer toxin database with an *E*-value ≤ 10^{-4} was examined to determine whether the alignment might imply possible toxicity of the query sequence. A summary of the BLASTP search parameters is provided in Table 138.

Results and Discussion

No alignments with an E-value ≤ 10^{-4} were returned between the IPD072Aa protein sequence and any protein sequence in the DuPont Pioneer toxin database (Figure 57). Therefore, no toxicity concerns arose from the bioinformatics assessment of the IPD072Aa protein.

Conclusion

No alignments with an E-value ≤ 10^{-4} were returned between the IPD072Aa protein sequence and any protein sequence in the DuPont Pioneer toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the IPD072Aa protein.

Table 140. Summary of BLASTP Parameters Used

BLAST Version	Program	Low-Complexity Filter	*E*-value Cutoff	Matrix	Gap Existence Penalty	Gap Extension Penalty	Maximum Alignments Returned
2.2.26	BLASTP	Off	10^{-4}	BLOSUM62 (Default)	11 (Default)	1 (Default)	Unlimited

Figure 56. Amino Acid Sequence of the IPD072Aa Protein

```
  1   MGITVTNNSS NPIEVAINHW GSDGDTSFFS VGNGKQETWD RSDSRGFVLS

 51   LKKNGAQHPY YVQASSKIEV DNNAVKDQGR LIEPLS
```

Figure 57. Output from the BLASTP Search Between the IPD072Aa Protein Sequence and the DuPont Pioneer Toxin Database Sequences

```
BLASTP 2.2.26 (Sep-21-2011)



Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Reference for compositional score matrix adjustment: Altschul, Stephen F.,
John C. Wootton, E. Michael Gertz, Richa Agarwala, Aleksandr Morgulis,
Alejandro A. Schaffer, and Yi-Kuo Yu (2005) "Protein database searches
using compositionally adjusted substitution matrices", FEBS J. 272:5101-5109.

Query= IPD072aa
         (86 letters)

Database: toxins.txt
           7996 sequences; 1,263,199 total letters

Searching.................................................done

 ***** No hits found ******



  Database: toxins.txt
    Posted date:  Jan 10, 2019 12:01 PM
  Number of letters in database: 1,263,199
  Number of sequences in database:  7996
```

```
Lambda     K      H

   0.310    0.128    0.376


Gapped

Lambda     K      H

   0.267   0.0410    0.140



Matrix: BLOSUM62

Gap Penalties: Existence: 11, Extension: 1

Number of Sequences: 7996

Number of Hits to DB: 244,226

Number of extensions: 9279

Number of successful extensions: 16

Number of sequences better than 1.0e-04: 0

Number of HSP's gapped: 16

Number of HSP's successfully gapped: 0

Length of query: 86

Length of database: 1,263,199

Length adjustment: 50

Effective length of query: 36

Effective length of database: 863,399

Effective search space: 31082364

Effective search space used: 31082364

Neighboring words threshold: 11

Window for multiple hits: 40

X1: 16 ( 7.2 bits)

X2: 38 (14.6 bits)

X3: 64 (24.7 bits)

S1: 42 (21.7 bits)

S2: 88 (38.5 bits)
```

Materials and Methods for Characterization of PAT Protein from DP23211 Maize

Test Substance

The test substance consisted of PAT protein isolated from tissue derived from DP23211 maize. The whole plant tissue was collected at the V9 growth stage (the stage when the collar of the ninth leaf becomes visible; Abendroth et al., 2011) of development from plants grown at a Pioneer-owned field location (Johnston, IA, USA). The tissue was lyophilized, homogenized and stored at ≤ -50 °C, under study number PHI-2018-099.

Protein Extraction, Purification, and Concentration

The PAT protein was extracted from lyophilized maize tissue by homogenization with a Waring blender using chilled phosphate-buffered saline containing polysorbate 20 (PBST) extraction buffer (25 ml buffer per g tissue). The sample extract was then filtered through cheesecloth and clarified by centrifugation. Ammonium sulfate (AS) precipitation was used to further purify and concentrate the sample extract. Beginning at 0% AS saturation, AS was slowly added to the sample extract while stirring until 45% AS saturation was reached. The sample was centrifuged and the AS process was repeated with the supernatant, this time beginning at 45% AS saturation and progressing to 60%. The sample was centrifuged again and the fractionated pellets were solubilized in phosphate-buffered saline prior to running the samples through desalting columns. The eluted fractions were pooled and further purified by immunoaffinity chromatography. The immunoaffinity column was prepared by coupling a PAT protein mouse monoclonal antibody (2C10.D5.G8) to AminoLink Plus Coupling Gel. Elutions 2-5 from the immunoaffinity purification were pooled into one sample for further purification by ion exchange. The pooled sample was diluted 1:2 using 50 mM Tris pH 8 then added to the column containing Q Sepharose ion exchange resin. Eluted fractions were captured separately and then concentrated into one sample using a centrifugal concentrator (10K; Sartorius) and buffer exchanged to a volume of approximately 100 µl for the first purification run and 70 µl for the second purification run.

Following each purification, the final volume in the concentrator was estimated and 25% 4X NuPAGE LDS sample buffer and 10% 10X NuPAGE Sample Reducing Agent was added to the concentrated sample. The sample in the concentrator was heated for 2-5 minutes at 70-100 °C and then transferred to a microcentrifuge tube. The sample was then heat treated at 90-100 °C for 5 (±1) minutes and stored frozen at ≤ -10 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The LDS-treated sample stored at ≤ -10 °C was re-heated for 5 minutes at 90-100 °C and then loaded into 4-12% Bis-Tris gels. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into each gel to provide a visual verification that migration was within the range of the predicted molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES SDS running buffer and NuPAGE Antioxidant at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for N-terminal amino acid sequencing and peptide mapping.

For Coomassie staining, the gel was washed with ultrapure (American Society for Testing and Materials (ASTM) Type 1) water (referred to as water) 3 times for 5 minutes each and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, the gel was de-stained with water 4 times for at least 5 minutes each until the gel background was clear. Proteins were stained as blue-colored bands on the gel. The gel image was captured electronically using an imaging system (Bio-Rad ChemiDoc MP).

Western Blot Analysis

Following SDS-PAGE, the resulting gel was assembled into a nitrocellulose (NC) iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gel to the NC membrane for 7 minutes with a pre-set program (P3).

Following protein transfer, the membrane was blocked in PBST containing 5% weight/volume (w/v) non-fat dry milk for 60 minutes at ambient laboratory temperature. Before and after the blocking step, the membrane was washed with PBST 3 times for 1 minute each to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with an PAT monoclonal antibody 22H2.G4 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk. Following primary antibody incubation, the membrane was washed 4 times in PBST for 5 minutes each. The membrane was incubated for 60 minutes at ambient laboratory temperature with a secondary antibody (anti-mouse IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% non-fat dry milk. The membrane was washed 4 times with PBST for 5 minutes each. The blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system (Bio-Rad ChemiDoc MP).

Materials and Methods for Characterization of PMI Protein from DP23211 Maize

Test Substance

The test substance consisted of PMI protein isolated from tissue derived from DP23211 maize. The whole plant tissue was collected at the V9 growth stage (the stage when the collar of the ninth leaf becomes visible; Abendroth et al., 2011) of development from plants grown at a Pioneer-owned field location (Johnston, IA, USA). The tissue was lyophilized, homogenized and stored at ≤-50 °C, under study number PHI-2018-099.

Protein Extraction, Purification, and Concentration

The PMI protein was extracted from lyophilized maize tissue by homogenization with a Waring blender using chilled phosphate-buffered saline containing polysorbate 20 (PBST) extraction buffer with EDTA-free Complete Protease Inhibitors (20 ml buffer per g tissue). The sample extract was then filtered through cheesecloth, clarified by centrifugation, filtered through a 0.45 µm PES vacuum filter unit, and fractionated using ammonium sulfate (AS) precipitation. Beginning at 0% AS saturation, AS was slowly added to the sample extract while stirring until 45% AS saturation was reached. The sample was centrifuged and the AS process was repeated with the supernatant, this time beginning at 45% AS saturation and progressing to 60%. The sample was centrifuged again and the fractionated pellet was solubilized in phosphate-buffered saline prior to running the sample through a desalting column. The eluted fraction was further purified by immunoaffinity chromatography. The immunoaffinity column was prepared by coupling rabbit polyclonal antibody (R164) anti-PMI to AminoLink Plus Coupling Gel. Elutions 2-4 from the immunoaffinity purification were concentrated into one sample using a centrifugal concentrator (Microsep 30K; Pall Life Sciences) and buffer exchanged to a volume of approximately 100 µl.

Following extraction, purification, and concentration, the final volume in the concentrator was estimated and an equal volume of 2X NuPAGE LDS sample buffer with Reducing Agent was added to the concentrated sample. The sample in the concentrator was heated for 2-5 minutes at 70-100 °C and then transferred to a microcentrifuge tube. The sample was then heat treated at 90-100 °C for 5 (±1) minutes and stored frozen at < -10 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The LDS treated sample stored at ≤ -10 °C was re-heated for 5 minutes at 90-100 °C and then loaded into 4-12% Bis-Tris gels. Prestained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into each gel to provide a visual verification that migration was within the range of the predicted molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES SDS running buffer and NuPAGE Antioxidant at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for peptide mapping.

For Coomassie staining, the gel was washed with ultrapure (American Society for Testing and Materials (ASTM) Type 1) water (referred to as water) 3 times for 5 minutes each and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, the gel was de-stained with water 4 times for at least 5 minutes each until the gel background was clear. Proteins were stained as blue-colored bands on the gel. The gel image was captured electronically using an imaging system (Bio-Rad ChemiDoc MP).

Western Blot Analysis

Following SDS-PAGE, the resulting gel was assembled into a nitrocellulose (NC) iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gel to the NC membrane for 7 minutes with a pre-set program (P3).

Following protein transfer, the membrane was blocked in PBST containing 5% weight/volume (w/v) non-fat dry milk for 60 minutes at ambient laboratory temperature. Before and after the blocking step, the membrane was washed with PBST for 1 minute to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with a PMI monoclonal antibody (13D11.F11.C12) conjugated to horseradish peroxidase diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk. Following primary antibody incubation, the membrane was washed 3 times in PBST for 5 minutes each. The blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

Appendix 6. Methods for Determination of DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI Protein Concentrations

A field trial was conducted during the 2018 growing season at six sites in commercial maize-growing regions of the United States (one site in each of Iowa, Illinois, Indianan, Minnesota, and Pennsylvania) and Canada (one site in Ontario). A randomized complete block design with four blocks was utilized at each site. Procedures employed to control the introduction of experimental bias included the use of non-systematic selection of trial and plot areas within each site, randomization of maize entries within each block, and uniform maintenance treatments across each plot area.

Sample Collection

The following tissue samples were collected: Root (V6, V9, R1, R4, and R6 growth stage), leaf (V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), forage (R4 growth stage at five sites and R4-R5 growth stage at remaining site), whole plant (R1 and R6 growth stages), and grain (R6 growth stage). Growth stages are described in Table 139. One sample per plot was collected for each tissue set. All samples were collected from impartially selected, healthy, representative plants to minimize potential bias.

Table 141. Maize Growth Stage Descriptions

Growth Stage	Description
V6	The stage when the collar of the sixth leaf becomes visible.
V9	The stage when the collar of the ninth leaf becomes visible.
R1	The stage when silks become visible.
R4	The stage when the material within the kernel produces a doughy consistency.
R5	The stage when all or nearly all the kernels are dented or denting.
R6	Typical grain harvest would occur. This stage is regarded as physiological maturity.

Note: Growth stages (Abendroth *et al.,* 2011).

Samples were collected as follows:

- Each root sample was obtained by cutting a circle 10-15 in. (25-38 cm) in diameter around the base of the plant to a depth of 7-9 in. (18-23 cm). The roots were thoroughly cleaned with water and removed from the plant. No above ground brace roots were included in the sample. The root tissue was cut into sections of 1 in. (2.5 cm) or less in length and collected to fill no more than 50% of a pre-labeled, 50-ml vial.

- Each leaf sample was obtained by pruning the youngest, healthy leaf that had emerged at least 8 in. (20 cm) from the whorl of the plant. The tissue was cut into sections of 1 in. (2.5 cm) or less in length and collected into a pre-labeled, 50-ml vial.

- Each pollen sample was obtained by bagging and shaking a selected tassel to dislodge the pollen. The tassel selected for sampling had one-half to three-quarters of the tassel's main spike shedding pollen. For some plots, pollen may have been pooled from multiple plants within the same plot in order to collect the appropriate amount. The pollen was screened for anthers and foreign material, and then collected in a pre-labeled, 35-ml vial.

- Each whole plant sample was obtained by cutting the plants approximately 4-6 in. (10-15 cm) above the soil surface line. The stalks and ears (R1), or husks and cobs (R6) were chopped into sections of 3 in. (7.6 cm) or less in length and the leaves were cut into sections of 12 in. (30 cm) or less in length and collected into a pre-labeled, plastic-lined, cloth bag. The plants selected for sampling at the R1 growth stage contained ears that were covered prior to silking. The plants selected for sampling at the R6 growth stage contained self-pollinated ears. Any secondary or tertiary ears with exposed silks were removed from the plants selected for sampling. The R6 whole plant sample did not contain grain; kernels were removed from the cob and collected for grain sampling.

- Each forage sample was obtained by cutting one plant approximately 4-6 in. (10-15 cm) above the soil surface line. The stalk and ear were chopped into sections of 3 in. (7.6 cm) or less in length and the leaves were cut into sections of 12 in. (30 cm) or less in length and collected into a pre-labeled, plastic-lined, cloth bag. The plants selected for forage sampling contained self-pollinated ears.

- Each grain sample was obtained by husking and shelling the grain from one selected ear. Each ear selected for sampling was a primary ear that had previously been self-pollinated. For each sample, a representative sub-sample of 15 kernels was collected into an individual pre-labeled vial.

Sample Processing, Shipping, and Storage

Each sample was uniquely labeled with a sample identification number and barcode for sample tracking by site, entry, block, tissue, and growth stage. Samples were placed on dry ice immediately after collection in the field and stored on dry ice until shipment. Samples were then shipped frozen on dry ice to Pioneer Hi-Bred International, Inc. for processing and analysis. Upon arrival, samples were stored frozen (-80 °C freezer unit).

For the samples for DvSSJ1 dsRNA analysis, pollen samples were stored frozen (-80 °C freezer unit) until analysis and root, leaf, forage, whole plant, and grain samples were finely homogenized and stored frozen (-80°C freezer unit) until analysis.

For the samples for IPD072Aa, PAT, and PMI protein analysis, forage and whole plant samples were coarsely homogenized on dry ice prior to lyophilization. All samples were lyophilized under vacuum until dry. Following lyophilization, pollen samples were stored frozen (-20 °C freezer unit) until analysis and root, leaf, forage, whole plant, and grain samples were finely homogenized and stored frozen (-20°C freezer unit) until analysis.

DvSSJ1 dsRNA Concentration Determination

Sample Weighing

Tissue sub-samples were weighed at the following target fresh weight ranges: 9-12 mg for pollen; 15-20 mg for leaf; 28-35 mg for grain, 24-30 mg for forage, and 40-50 mg for root and whole plant. Minor fluctuations outside of the weight ranges were allowed, as results were calculated per gram of fresh tissue.

Total RNA Extraction

Total RNA was extracted by adding 500 µl of RNA lysis buffer (50 mM sodium citrate pH 4.5, 25 mM EDTA, 75 mM sodium chloride, 1% sodium dodecyl sulfate, and 1% β-mercaptoethanol freshly added) to pre-weighed tissue samples. An equal volume (500 µl) of acid-phenol:chloroform mixture (1:1) was added to the slurry and samples were ground with 1.0 mm zirconium oxide beads. After grinding, samples were incubated for 5 minutes at approximately 65 °C then centrifuged in Phase Lock Gel-Heavy tubes to separate the phases. The aqueous phase was removed, transferred to fresh tubes, and 1.5 ml TRI-reagent and 2 ml 200-Proof Ethanol were added. The total RNA was purified using Zymo Direct-zol RNA Miniprep Plus Kit and eluted from the column in 100 µl of RNase-free water. The concentration of total RNA was determined using a NanoDrop 2000 UV-Vis Spectrophotometer. Representative samples were checked for RNA quality and integrity by gel electrophoresis. Samples that showed poor RNA quality were

excluded from DvSSJ1 dsRNA concentration analysis. Total RNA was stored frozen (-80 °C freezer unit) until QuantiGene analysis.

Total RNA yield was calculated as follows:

$$Total\ RNA\ Concentration\ \frac{ng}{\mu l}\ x\ 100\ \mu l = Total\ RNA\ Yield\ (ng)$$

QuantiGene Analysis

QuantiGene analysis was used to determine the concentration of DvSSJ1 dsRNA in tissues derived from DP23211 maize. The QuantiGene method was internally validated to demonstrate method suitability.

The DvSSJ1 QuantiGene Plex Assay method utilized magnetic beads specific to the target to measure the amount of DvSSJ1 dsRNA in samples. Prior to analysis, total RNA was diluted to 10 – 30 ng total RNA/µl (equal to 200 – 600 ng total RNA/well) in QuantiGene Homogenizing Solution (QHS). Standards and samples (both typically analyzed in triplicate wells) were first denatured (95 °C for five minutes) and annealed (55 °C) in a 96-well PCR plate with a sequence-specific probe set designed by Affymetrix that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The mixture of sample and probe set was transferred to a hybridization plate containing bead mix (100 µl total volume) for overnight hybridization at approximately 54 °C. Following hybridization, a magnetic separation device was used to wash unbound substances from the plate. Signal was amplified on the LE probes by sequential incubations (each 1 – 2 hours at approximately 50 °C) with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Each well was then incubated with the fluorescent protein streptavidin phycoerythrin (SAPE; 30 minutes at room temperature) and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v4.2 software.

Calculations for Determining DvSSJ1 dsRNA Concentration (Fresh Weight)

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the MFI values obtained for each set of sample wells to a DvSSJ1 dsRNA concentration value.

A seven-point standard curve was included on each plate (2.45×10^{-6} – 6.00×10^{-4} µg/ml). The standard curve was prepared by diluting the reference substance in 20 ng total RNA/µl (equal to 400 ng total RNA/well) extracted from conventional maize leaf tissue that did not contain DvSSJ1 (diluted in QHS). The standard curve diluent (referred to as blank) was also loaded to each plate and the average of the blank was subtracted from all wells on the plate.

The Log-Log fit was used to generate the standard curve and was applied as follows:

$$Log(y) = A + B * Log(x)$$

where x = known standard concentration (pg/well) and y = respective MFI value

Interpolation of the sample concentration (pg/well) was performed by solving for x in the above equation using the values for A and B that were determined for the standard curve.

The QuantiGene result (pg DvSSJ1 dsRNA/well) was divided by the amount of total RNA loaded to each well, for example:

$$\frac{pg\ DvSSJ1\ dsRNA}{well}\ x\frac{well}{400\ ng\ Total\ RNA} = \frac{pg\ DvSSJ1\ dsRNA}{ng\ Total\ RNA}$$

To calculate the concentration of DvSSJ1 dsRNA per gram tissue fresh weight for each sample, the amount of DvSSJ1 dsRNA per ng total RNA was multiplied by the total RNA yield from that sample, and divided by the sample fresh weight:

$$\frac{pg\ DvSSJ1\ dsRNA}{ng\ Total\ RNA}x\frac{Total\ RNA\ Yield\ (ng)}{Sample\ Weight\ (mg)}x\frac{1}{1000} = \frac{\mu g\ DvSSJ1\ dsRNA}{g\ tissue}$$

Calculations for Determining DvSSJ1 dsRNA Concentrations (Dry Weight)

A sub-sample was collected from each tissue sample and lyophilized. Weights were recorded before and after lyophilization to calculate fresh weight to dry weight ratio (FW:DW) for each sample. The FW:DW ratio was then multiplied by the DvSSJ1 dsRNA fresh weight expression value to generate DvSSJ1 dsRNA expression values on a dry weight basis.

IPD072Aa, PAT, and PMI Protein Concentration Determination

The concentrations of IPD072Aa, PAT, and PMI proteins were determined using quantitative enzyme-linked immunosorbent assay (ELISA) methods that have been internally validated to demonstrate method suitability.

Processed tissue sub-samples were weighed at the following target weights: 5 mg for pollen; 10 mg for leaf; 20 mg for grain and root; and 30 mg for forage. IPD072Aa protein leaf, pollen, whole plant, forage, and grain samples were extracted with 0.60 ml of chilled 25% StabilZyme Select in phosphate-buffered saline containing polysorbate 20 (PBST), and root samples were extracted in chilled H5 buffer (comprised of 90 mM HEPES, 140 mM sodium chloride, 1.0% polyethylene glycol, 1.0% PVP-40, 1.0% bovine serum albumin, 0.007% thimerosal, and 0.3% polysorbate 20). PAT and PMI protein samples were extracted with 0.60 ml of chilled phosphate-buffered saline

containing polysorbate 20 (PBST). All extracted samples were centrifuged, and then supernatants were removed and prepared for analysis.

ELISA methods were performed as follows:

- *IPD072Aa Protein ELISA Method*: Prior to analysis, samples were diluted as applicable with 25% StabilZyme Select in PBST. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with an IPD072Aa specific antibody. Following incubation, unbound substances were washed from the plate and the bound IPD072Aa protein was incubated with a different IPD072Aa specific antibody conjugated to the enzyme horseradish peroxidase (HRP). Unbound substances were washed from the plate. Detection of the bound IPD072Aa antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader.

- *PAT Protein ELISA Method*: Prior to analysis, samples were diluted as applicable in PBST. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were co-incubated with a PAT specific antibody conjugated to the enzyme HRP in a plate pre-coated with a different PAT specific antibody. Following incubation, unbound substances were washed from the plate. Detection of the bound PAT antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the OD of each well was determined using a plate reader.

- *PMI ELISA Method*: Prior to analysis, samples were diluted as applicable in PBST. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in plate pre-coated with a PMI-specific antibody. Following incubation, unbound substances were washed from the plate and the bound PMI protein was incubated with a different PMI-specific antibody conjugated to the enzyme HRP. Unbound substances were washed from the plate. Detection of the bound PMI-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the OD of each well was determined using a plate reader.

Calculations for Determining IPD072Aa, PAT, and PMI Protein Concentrations

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the OD values obtained for each set of sample wells to a protein concentration value.

A standard curve was included on each ELISA plate. The equation for the standard curve was derived by the software, which used a quadratic fit to relate the OD values obtained for each set of standard wells to the respective standard concentration (ng/ml).

The quadratic regression equation was applied as follows: $y = Cx^2 + Bx + A$

where x = known standard concentration and y = respective absorbance value (OD)

Interpolation of the sample concentration (ng/ml) was performed by solving for x in the above equation using the values for A, B, and C that were determined for the standard curve.

$$\text{Sample Concentration (ng/ml)} = \frac{-B + \sqrt{B^2 - 4C(A - \text{sample OD})}}{2C}$$

For example, given curve parameters of A = 0.0476, B = 0.4556, C= -0.01910, and a sample OD = 1.438

$$\text{Sample Concentration} = \frac{-0.4556 + \sqrt{0.4556^2 - 4(-0.01910)(0.0476 - 1.438)}}{2(-0.01910)} = 3.6 \text{ ng/ml}$$

The sample concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the interpolated concentration by N.

Adjusted Concentration = Interpolated Sample Concentration x Dilution Factor

For example, given an interpolated concentration of 3.6 ng/ml and a dilution factor of 1:20

Adjusted Concentration = 3.6 ng/ml x 20 = 72 ng/ml

Adjusted sample concentration values obtained from SoftMax Pro GxP software were converted from ng/ml to ng/mg sample weight as follows:

$$\text{Sample Concentration (ng protein/mg sample weight)} = \text{Sample Concentration (ng/ml)} \times \frac{\text{Extraction Buffer Volume (ml)}}{\text{Sample Target Weight (mg)}}$$

For example, sample concentration = 72 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 10 mg

$$\text{Sample Concentration (ng protein/mg sample weight)} = 72 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{10 \text{ mg}} = 4.3 \text{ ng/mg}$$

The reportable assay lower limit of quantification (LLOQ) in ng/ml was calculated as follows:

Reportable Assay LLOQ (ng/ml) = (lowest standard concentration - 10%) x minimum dilution

For example, lowest standard concentration = 0.50 ng/ml and minimum dilution = 10

Reportable Assay LLOQ (ng/ml) = (0.50 ng/ml - (0.50 x 0.10)) x 10 = 4.5 ng/ml

The LLOQ, in ng/mg sample weight, was calculated as follows:

$$\text{LLOQ} = \text{Reportable Assay LLOQ (ng/ml)} \times \frac{\text{Extraction Buffer Volume (ml)}}{\text{Sample Target Weight (mg)}}$$

For example, reportable assay LLOQ = 4.5 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 10 mg

$$\text{LLOQ} = 4.5 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{10 \text{ mg}} = 0.27 \text{ ng/mg sample weight}$$

Statistical Analysis

Statistical analysis of the DvSSJ1 dsRNA concentration results consisted of the calculations of means, ranges, and standard deviations on fresh weight and dry weight basis.

Statistical analysis of the IPD072Aa, PAT, and PMI protein concentration results consisted of the calculations of means, ranges, and standard deviations. Individual sample results below the LLOQ were assigned a value equal to half of the LLOQ for calculation purposes.

Appendix 7. Bioinformatics Methods Used to Analyze Sequence Comparison of *ssj1* Homologs

Materials and Methods

The sequences of the *ssj1* homologs from 13 organisms with varied evolutionary distance from WCR were obtained from published data (Hu *et al.*, 2016)(Table 38).

The transcriptomes of six additional insects, including the CDM, MBD, MWM, PL, RVB, and SWM were assembled as previously described (Niu *et al.*, 2017) (Table 38). Briefly, complementary DNAs (cDNAs) prepared from larvae of each species were sequenced by Illumina paired-end and 454 Titanium sequencing technologies. *De novo* transcriptome assemblies were performed using the Trinity method (Grabherr *et al.*, 2011). The coding sequence of *dvssj1* gene sequence (Accession number: KU562965) was used for tblastx search transcriptome assembly. The AlignX tool of Vector NTi (Invitrogen) was used to create alignments between the 210-bp *dvssj1* sequence and the *ssj1* homologous sequences from each insect. Each alignment was inspected to determine the nucleotide percent identity over the length of the alignment. The longest contiguous match between the 210-bp *dvssj1* sequence and each of the homologous sequences from the various insects was determined using a Perl script created by Corteva Agriscience (findExactMatches.pl).

Results

Using *in silico* analysis, the closest sequence match (percent identity to the 210-bp *dvssj1* sequence) was the *ssj1* homologous gene from WCR, which as intended had a 100% sequence match, 0 SNPs, and 190 21-nt matches (Table 38). The *ssj1* homologous gene from the closely related species, NCR, shared 97.1% identity with the 210-bp *dvssj1* sequence, with 6 SNPs and 135 21-nt matches. The *ssj1* homologous gene from SCR shared 92.9% identity with the 210-bp *dvssj1* sequence, with 15 SNPs and 79 21-nt matches. The *ssj1* homologous genes from the other Coleoptera within the family Chrysomelidae (CFB, SFB, and CPB) as well as species within the family Tenebrionidae, the family Coccinellidae, and the family Staphylinidae had decreasing percent identity with the 210-bp *dvssj1* sequence, ranging from 77.6 to 61.9% similarity and an increasing number of SNPs (ranging from 47 to 80). All Lepidoptera species within the four families, as well as the honey bee (*Apis mellifera)* and the insidious flower bug (*Orius insidiosus)* also had lower percent identity with the 210-bp *dvssj1* sequence, ranging from 68.1 to 60% similarity, and an increased number of SNPs (ranging from 67 to 84).

The bioinformatics analysis of DvSSJ1 dsRNA shows that the 210-bp sequence was most closely aligned with WCR as expected, and showed decreasing levels of complementarity with increasing phylogenetic distance from WCR, with no contiguous 21nt matches identified outside of the genus *Diabrotica*.

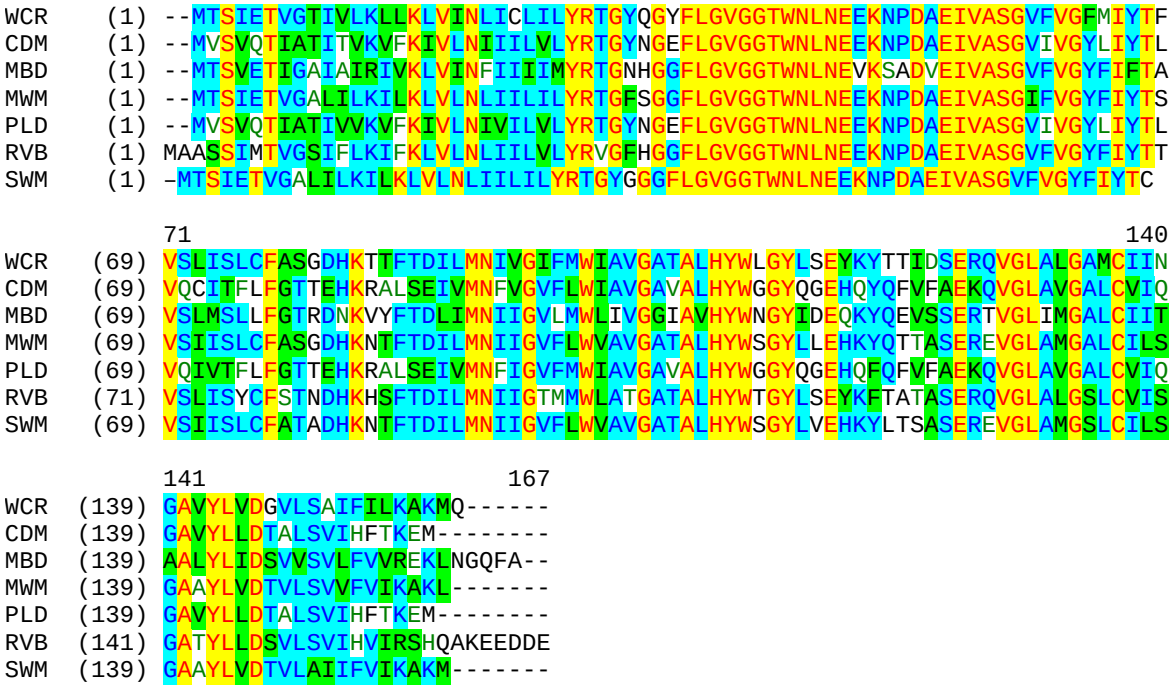


Figure 58. Amino Acid Sequence Alignment of the DvSSJ1 Protein and its Homologs from Six Species

The coding sequence of the *dvssj1* gene (KU562965) was used for tblastx search transcriptome assembly. The amino acid sequence of the DvSSJ1 protein (WCR) was used to align with the SSJ1 protein homologs from six insects. Nucleotide sequences of the six insects were used for sequence analyses (Table 38; including CM, MBD, MWM, PL, RVB and SWM).

Appendix 8. Materials and Methods for Human Dietary Exposure Evaluation

Materials

Maize grain samples were analyzed for DvSSJ1 dsRNA and expressed IPD072Aa, PAT, and PMI protein concentrations. The mean concentrations of DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI proteins in DP23211 maize grain were 0.00413, 2.1, 5.1, and 4.3 mg/kg dry weight, respectively.

Methods

The dietary exposures to DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI proteins were estimated using the DEEM™ - FCID program. Annual mean and 95th percentile daily exposures were calculated for the U.S. population and several sub-populations for a conservative hypothetical total replacement scenario (100% DP23211 maize inclusion), assuming that all maize products consumed are derived from DP23211 maize grain.

DEEM™ - FCID categorizes maize (field corn) consumption as: flour, flour-baby food, meal, meal-baby food, bran, starch, starch-baby food, syrup, syrup-baby food, oil, and oil-baby food. For the purposes of this exposure assessment, it was assumed that corn flour, corn flour-baby food, corn meal, corn meal-baby food, and corn bran contain DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI proteins at levels equivalent to the mean concentrations in grain from PHI-2018-010 and that there is no degradation of expressed proteins or dsRNA during processing or cooking of foodstuffs in those categories. Because the processing conditions would denature and remove virtually all proteins in corn starch, corn starch-baby food, corn syrup, corn syrup-baby food, corn oil, and corn oil-baby food (CRA, 2006a; CRA, 2006b; CRA, 2006c; Hefle and Taylor, 1999), consumption of these foodstuffs was not considered for exposure estimates.

Dietary risk was assessed by 1) evaluating the estimated DvSSJ1 dsRNA exposure utilizing the Threshold of Toxicological Concern (TTC) method for assessment of substances of unknown toxicity found in food (Kroes et al., 2005), and 2) calculating the margin of exposures (MOE) for IPD072Aa, PAT, and PMI proteins by dividing the respective protein dose administered in corresponding 14-day acute toxicity studies[a] where no adverse effects were observed by the highest respective acute dietary exposure estimates.

Appendix 9. Materials and Methods for Livestock Dietary Exposure

Materials

Maize grain and forage samples were analyzed for the DvSSJ1 dsRNA and expressed IPD072Aa, PAT, and PMI protein concentrations. The mean concentrations of DvSSJ1 dsRNA in DP23211 maize grain and forage were 0.00413 and 0.0190 mg/kg dry weight, respectively. The mean concentrations of IPD072Aa, PAT, and PMI proteins in DP23211 maize grain were 2.1, 5.1, and 4.3 mg/kg dry weight, respectively and the mean concentrations of IPD072Aa, PAT, and PMI proteins in DP23211 maize forage were 16, 8.2, and 9.4 mg/kg dry weight, respectively.

Methods

Daily dietary exposure (DDE) to DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI proteins from DP23211 maize grain and forage were calculated for various livestock species using estimates of animal body weight (BW), daily feed intake, and grain and/or forage/silage inclusion rates specific for North America (OECD, 2013; corn, field-grain or corn, field-forage/silage). The following conservative total replacement scenarios were utilized:

- 100% DP23211 maize grain replacement for poultry (broiler, layer, turkey), swine (breeding, finishing), cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize forage/silage replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP23211 maize grain and forage/silage combination replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb)

Margin of exposure (MOE) assessments were made by comparing the conservatively estimated DDEs (assuming all maize grain and/or forage/silage in the diet derived from DP23211 maize) of each expressed protein with corresponding doses administered in acute oral toxicity studies with mice where no treatment-related effects were observed on parameters such as body weights and other clinical observations.

Refer to Table 140 for daily dietary intake of maize grain by the various livestock species.

Table 142. Maize Forage/Silage and Grain Consumption by Various Livestock

Animal		BW (kg)[a]	Total Daily Feed Intake (kg DM/Animal)[a]	Corn, Field – Forage/Silage Inclusion Rate (DM)[a]	Corn Forage/Silage Daily Dietary Intake (g DM Feed/kg BW)[b]	Corn, Field – Grain Inclusion Rate (DM)[a]	Corn Grain Daily Dietary Intake (g DM Feed/kg BW)[b]
Poultry	Broiler	2	0.16	*	*	75%	60.0
	Layer	1.9	0.12	*	*	75%	47.4
	Turkey	8	0.5	*	*	75%	46.9
Swine	Breeding	270	2	*	*	85%	6.3
	Finishing	100	3.1	*	*	85%	26.4
Cattle	Beef	500	9.1	15%	2.7	80%	14.6
	Dairy	600	24	45%	18.0	45%	18.0
Sheep	Ram/Ewe	85	2	45%	10.6	45%	10.6
	Lamb	40	1.5	45%	16.9	45%	16.9

Notes: * Not used or is minor feedstuff (<5% of diet); BW = body weight, DM = dry matter

[a] CAN/US-specific values for animal body weight, feed consumption and feedstuff inclusion rates are from OECD (2013). The inclusion rates represent the proportion of daily ration on a DM basis.

[b] Maize forage/silage or grain daily dietary intake (g DM feed/kg BW) = (Total daily feed intake (kg DM/animal) / BW (kg)) x (1000 g/1 kg) x (forage/silage or grain inclusion rate (%).

Appendix 10. Materials and Methods for Nutrient Composition Assessment

Field Trial Experimental Design

A field trial was conducted during the 2018 growing season at eight sites in commercial maize-growing regions of the United States (one site in Iowa, Indiana, Minnesota, Pennsylvania, Texas, and two sites in Illinois) and Canada (one site in Ontario). Each site included DP23211 maize, non-GE near-isoline control maize, and non-GE commercial reference maize. A randomized complete block design with four blocks was utilized at each site.

Sample Collection

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected from DP23211 maize, control maize, and reference maize lines. One sample per plot was collected and all samples were collected from impartially selected, healthy, representative plants. Each sample was uniquely labeled with a sample identification number and barcode for sample tracking, and is traceable by site, entry, block, tissue, and growth stage.

Each forage sample was obtained by cutting the aerial portion of the plants from the root system approximately 4-6 in. (10-15 cm) above the soil surface. The plants were chopped into sections of 3 in. (7.6 cm) or less in length and approximately one-third of the chopped material was collected in a pre-labeled, plastic-lined, cloth bag.

Each grain sample was obtained at typical harvest maturity. The ears were husked and shelled, and the pooled grain was collected into a large pre-labeled, plastic, resealable bag and then placed into a pre-labeled, plastic-lined, cloth bag.

Each forage and grain sample was placed in chilled storage (*e.g.*, coolers with wet ice, artificial ice, dry ice, or in a freezer), then transferred to a freezer (≤ -10 °C). Samples were shipped frozen to Pioneer Hi-Bred International, Inc., and then shipped frozen to EPL Bio Analytical Services (EPL BAS, Niantic, IL, USA) for analyses or shipped frozen directly to EPL BAS.

Nutrient Composition Analyses

Nutrient composition analyses of forage and grain samples were conducted by EPL BAS. All procedures and methods used by EPL BAS are described in Table 141. Nutrient composition analyses of forage and grain samples included the determination of the following analytes:

Proximates, Fiber, and Minerals Composition in Forage

- Moisture*
- Crude Protein
- Crude Fat
- Crude Fiber
- Acid Detergent Fiber (ADF)
- Neutral Detergent Fiber (NDF)
- Ash
- Carbohydrates
- Calcium
- Phosphorus

Note: Moisture data were used to convert corresponding analyte values for a given sample to a dry weight basis, and were not included in subsequent statistical analysis and reporting of results.

Proximates and Fiber Composition in Grain

- Moisture*
- Total Dietary Fiber
- Crude Protein
- Crude Fat
- Crude Fiber
- Acid Detergent Fiber (ADF)
- Neutral Detergent Fiber (NDF)
- Ash
- Carbohydrates

*Note: Moisture data were used to convert corresponding analyte values for a given sample to a dry weight basis, and were not included in subsequent statistical analysis and reporting of results.

Fatty Acids Composition in Grain

- Lauric Acid (C12:0)
- Myristic Acid (C14:0)
- Palmitic Acid (C16:0)
- Palmitoleic Acid (C16:1)
- Heptadecanoic Acid (C17:0)
- Heptadecenoic Acid (C17:1)
- Stearic Acid (C18:0)
- Oleic Acid (C18:1)
- Linoleic Acid (C18:2)
- α-Linolenic Acid (C18:3)
- Arachidic Acid (C20:0)
- Eicosenoic Acid (C20:1)
- Eicosadienoic Acid (C20:2)
- Behenic Acid (C22:0)
- Erucic Acid (C22:1)
- Lignoceric Acid (C24:0)

Amino Acids Composition in Grain

- Alanine
- Arginine
- Aspartic Acid
- Cystine
- Glutamic Acid
- Glycine
- Histidine
- Isoleucine
- Leucine
- Lysine
- Methionine
- Phenylalanine
- Proline
- Serine
- Threonine
- Tryptophan
- Tyrosine
- Valine

Minerals Composition in Grain

- Calcium
- Copper
- Iron
- Magnesium
- Manganese
- Phosphorus
- Potassium
- Sodium
- Zinc

Vitamins Composition in Grain

- β-Carotene
- Vitamin B1 (Thiamine)
- Vitamin B2 (Riboflavin)
- Vitamin B3 (Niacin)
- Vitamin B5 (Pantothenic Acid)
- Vitamin B6 (Pyridoxine)
- Vitamin B9 (Folic Acid)
- α-Tocopherol
- β-Tocopherol
- γ-Tocopherol
- δ-Tocopherol

Note: an additional analyte, Total Tocopherols, was subsequently calculated as the sum of the α-, β-, γ-, and δ-tocopherol values for each sample for use in statistical analysis and reporting of results.

Secondary Metabolites and Anti-Nutrients Composition in Grain

- *p*-Coumaric Acid
- Ferulic Acid
- Furfural
- Inositol
- Phytic Acid
- Raffinose
- Trypsin Inhibitor

Table 143. Methods for Compositional Analysis of DP23211 Maize

Nutritional Analyte	Method
Moisture Forage and Grain	The analytical procedure for moisture determination was based on a method published by the AOAC International. Samples were assayed to determine the percentage of moisture by gravimetric measurement of weight loss after drying in a forced air oven (forage) and a vacuum oven (grain).
Ash Forage and Grain	The analytical procedure for ash determination was based on a method published by the AOAC International. Samples were analyzed to determine the percentage of ash by gravimetric measurement of the weight loss after ignition in a muffle furnace.
Crude Protein Forage and Grain	The analytical procedure for crude protein determination utilized an automated Kjeldahl technique based on a method provided by the manufacturer of the titrator unit (Foss-Tecator) and the AOAC International. Ground samples were digested in the presence of a catalyst. The digestate was then distilled and titrated with a Foss-Tecator Kjeltec Analyzer unit.
Crude Fat Forage and Grain	The analytical procedure for crude fat determination was based on methods provided by the American Oil Chemists' Society (AOCS) and the manufacturer of the hydrolysis and extraction apparatus (Ankom Technology). Samples were hydrolyzed with 3N hydrochloric acid at 90 °C for 80 minutes for forage and 60 minutes for grain. The hydrolysates were extracted with a petroleum ether/ethyl ether/ethyl alcohol solution at 90 °C for 60 minutes. The ether extracts were evaporated and the fat residue remaining determined gravimetrically.
Carbohydrates Forage and Grain	The carbohydrate content in maize forage and grain on a dry weight basis was calculated using a formula obtained from the United States Department of Agriculture "*Energy Value of Foods,*" in which the percent dry weight of crude protein, crude fat, and ash was subtracted from 100%.
Crude Fiber Forage and Grain	The analytical procedure for crude fiber determination was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC International, and the AOCS. Samples were analyzed to determine the percentage of crude fiber by digestion and solubilization of other materials present.
Neutral Detergent Fiber Forage and Grain	The analytical procedure for neutral detergent fiber (NDF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC International, and the *Journal of AOAC International*. Samples were analyzed to determine the percentage of NDF by digesting with a neutral detergent solution, sodium sulfite, and alpha amylase. The remaining residue was dried and weighed to determine the NDF content.
Acid Detergent Fiber Forage and Grain	The analytical procedure for acid detergent fiber (ADF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology) and the AOAC International. Samples were analyzed to determine the percentage of ADF by digesting with an acid detergent solution. The remaining residue was dried and weighed to determine the ADF content.

Table 141. Methods for Compositional Analysis of DP23211 Maize (continued)

Nutritional Analyte	Method
Total Dietary Fiber	The analytical procedure for the determination of total dietary fiber in grain was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC International, and the manufacturer of the protein titrator unit (Foss-Tecator). Duplicate samples were gelatinized with heat stable α-amylase, enzymatically digested with protease and amyloglucosidase to remove protein and starch, respectively, and then soluble dietary fiber precipitated with ethanol. The precipitate (residue) was quantified gravimetrically. Protein analysis was performed on one of the duplicate samples while the other duplicate sample was analyzed for ash. The weight of the protein and ash was subtracted from the weight of the residue divided by sample dry weight.
Minerals	The analytical procedure for the determination of minerals is based on methods published by the AOAC International and CEM Corporation. The maize forage minerals determined were calcium and phosphorus. Additional grain minerals determined were copper, iron, magnesium, manganese, potassium, sodium, and zinc. The samples were digested in a microwave based digestion system. Samples were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES).
Tryptophan	The analytical procedure for tryptophan determination was based on an established lithium hydroxide hydrolysis procedure with reverse phase ultra-performance liquid chromatography (UPLC) with ultraviolet (UV) detection published by the *Journal of Micronutrient Analysis*.
Cystine and Methionine	The analytical procedure for cystine and methionine determination was based on methods obtained from Waters Corporation, AOAC International, and *Journal of Chromatography A*. The procedure converts cystine to cysteic acid and methionine to methionine sulfone, after acid oxidation and hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives which are then analyzed by reverse phase UPLC with UV detection.
Additional Amino Acids	Along with tryptophan, cystine, and methionine, 15 additional amino acids were determined. The analytical procedure for analysis of these amino acids was based on methods obtained from Waters Corporation and the *Journal of Chromatography A*. The procedure converts the free acids, after acid hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives, which are analyzed by reverse phase UPLC with UV detection.
Fatty Acids	The analytical procedure for determination of fatty acids was based on methods published by the AOAC International and AOCS. The procedure converts the free acids, after ether extraction and base hydrolysis, to the fatty acid methyl ester (FAME) derivatives, which are analyzed by gas chromatography with flame ionization detection (GC/FID). Results are reported as percent total fatty acids but presented in the raw data as percent fresh weight.
Thiamine (Vitamin B1) and Riboflavin (Vitamin B2)	The analytical procedure for the determination of thiamine (vitamin B1) and riboflavin (vitamin B2) was based on a method published by the American Association of Cereal Chemists (AACC). The samples were extracted with 10% acetic acid/4.3% trichloroacetic acid solution. The samples were analyzed by reverse phase high pressure liquid chromatography (HPLC) tandem mass spectrometry (MS/MS).

Table 141. Methods for Compositional Analysis of DP23211 Maize (continued)

Nutritional Analyte	Method
Niacin (Vitamin B3)	The analytical procedure for the determination of niacin (vitamin B3) was based on a method published by the AACC. Niacin (vitamin B3) was extracted from the sample by adding deionized (DI) water and autoclaving. A tube array was prepared using three different dilutions of the samples. This tube array was inoculated with *Lactobacillus plantarum* and allowed to incubate for approximately 18 to 22 hours. After incubation, the bacterial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of nicotinic acid.
Pantothenic Acid (Vitamin B5)	The analytical procedure for the determination of pantothenic acid (vitamin B5) was based on a method from the AOAC International. Pantothenic acid (vitamin B5) was determined using a microbiological assay. Pantothenic acid (vitamin B5) was extracted from the sample by adding an acetic acid buffer solution and autoclaving. The pH was adjusted and a tube array was prepared using three different dilutions of the samples. This tube array was inoculated with *Lactobacillus plantarum* and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of D-pantothenic acid hemicalcium salt.
Pyridoxine (Vitamin B6)	The analytical procedure for the determination of pyridoxine (vitamin B6) was based on a method from the AACC. Pyridoxine (vitamin B6) was determined using a microbiological assay. Pyridoxine (vitamin B6) was extracted from the sample by adding sulfuric acid and autoclaving. The pH was adjusted and a tube array was prepared using four different dilutions of the samples. This tube array was inoculated with *Saccharomyces cerevisiae* and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 600 nm. The absorbance readings were compared to a standard curve generated using known concentrations of pyridoxine hydrochloride.
Total Folate as Folic Acid (Vitamin B9)	The analytical procedure for determination of total folate as folic acid was based on a microbiological assay published by the AACC. Samples were hydrolyzed and digested by protease and amylase enzymes to release the folate from the grain. A conjugase enzyme was used to convert the naturally occurring folypolyglutamates to folyldiglutamates. An aliquot of the extracted folates was mixed with a folate and folic acid free microbiological growth medium. The mixture was inoculated with *Lactobacillus casei*. The total folate content was determined by measuring the turbidity of the *Lactobacillus casei* growth response in the sample and comparing it to the turbidity of the growth response with folic acid standards using a spectrophotometer at 600 nm.

Table 141. Methods for Compositional Analysis of DP23211 Maize (continued)

Nutritional Analyte	Method
Total Tocopherols	The analytical procedure for determination of tocopherols was based on methods from the *Journal of the American Oil Chemists' Society* and *Analytical Sciences*. Alpha, beta, gamma, and delta tocopherols were extracted with hot hexane and the extracts were analyzed by normal phase UPLC with fluorescence detection.
Beta-Carotene	The analytical procedure for determination of beta-carotene was based on a method published by the AOAC International. Samples were extracted using a 40:60 acetone:hexane with tert-butylhydroquinone (TBHQ) solution then analyzed by HPLC-UV.
Trypsin Inhibitor	The analytical procedure for the determination of trypsin inhibitor was based on a method published by the AOCS. Trypsin inhibitor was extracted with sodium hydroxide. Trypsin was added to the extracts to react with trypsin inhibitor. The residual trypsin activity was measured with a spectrophotometer using the chromogenic trypsin substrate benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA). The amount of inhibitor was calculated based on the inhibition of trypsin activity.
Inositol and Raffinose	The analytical procedure for the determination of inositol and raffinose was based on a gas chromatography (GC) method published in the *Handbook of Analytical Derivatization Reactions*, an AACC method, and a method from the *Journal of Agricultural and Food Chemistry*. Extracted inositol was derivatized with butylboronic acid and analyzed by GC/FID. Raffinose was analyzed by reverse phase HPLC with refractive index detection.
Furfural	The analytical procedure for the determination of furfural was based on methods published in the *Journal of Agricultural and Food Chemistry*. Ground maize grain was analyzed for furfural content by reverse phase UPLC with UV detection.
p-Coumaric and Ferulic Acid	The analytical procedure for the determination of *p*-coumaric and ferulic acids was developed based on methods published in *Journal of Agricultural and Food Chemistry* and *The Journal of Chemical Ecology*. Ground maize grain was analyzed to determine the amounts of *p*-coumaric acid and ferulic acid by separating the total content of phenolic acids using reverse phase UPLC and UV detection.
Phytic Acid	The analytical procedure for the determination of phytic acid was based on a method published by the AOAC International. The samples were analyzed to determine the amount of phytic acid by extracting the phytic acid with dilute hydrochloric acid (HCl) and isolating it using an aminopropyl silica solid phase extraction column. Once isolated and eluted, the phytic acid was analyzed for elemental phosphorus by ICP-OES.

Statistical Analysis of Nutrient Composition Data

Statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA) to evaluate and compare the nutrient composition of forage and grain derived from DP23211 maize and the control maize.

Processing of Data

Values Below Lower Limit of Quantification

For statistical analysis, nutrient composition values reported as below the assay lower limit of quantification (LLOQ) were each assigned a value equal to half the respective LLOQ.

Conversion of Fatty Acid Assay Values

The raw data for all fatty acid analytes were provided by EPL Bioanalytical Services in units of percent fresh weight (%FW). Any fatty acid values below the %FW LLOQ were set to half the LLOQ value, and then all assay values were converted to units of % total fatty acids for statistical analyses.

For a given sample, the conversion to units of % total fatty acids were performed by dividing each fatty acid analyte value (%FW) by the total fresh weight of all fatty acids for that sample; for analyte values below the LLOQ, the half LLOQ value was used as the analyte value. Half LLOQ values were also included in the total fresh weight summations. After the conversion, a fixed LLOQ value was not available for a given individual fatty acid analyte on the % total fatty acids basis.

One fatty acid, erucic acid (C22:1), was excluded from the conversion and from statistical analyses because all sample values in the current study and in historical commercial reference maize lines were below the LLOQ.

Calculation of Total Tocopherol

One additional analyte (total tocopherol) was calculated for statistical analyses. The total amount of tocopherol for each sample was obtained by summing the assay values of α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol in the sample.

If the assay value of an individual analyte was below the LLOQ for a given sample, half of the LLOQ value was used in computing the total. The total was considered below the LLOQ only when all the individual analytes contributing to its calculation were below the LLOQ.

Selection of Statistical Method

For a given analyte, the number of samples below the assay LLOQ value determined how the statistical analyses were conducted. The following rules were implemented:

If both DP23211 maize and the control maize had < 50% of samples across sites below the LLOQ, then mixed model was applied in the across-site analysis.

If either DP23211 maize or the control maize had ≥ 50% samples below the LLOQ, but not both entries had 100% of samples below the LLOQ across sites, then Fisher's exact test would be conducted. The Fisher's exact test assessed whether there was a significant difference (P-value < 0.05) in the proportion of samples below the LLOQ between these two maize lines across sites.

If both DP23211 maize and the control maize had 100% of samples below the LLOQ, then statistical analyses were not performed.

Statistical Model for Across-Site Analysis

For a given analyte, data were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ Model 1

$\ell_j \sim iid\ N(0, \sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0, \sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Ent\times Site})$, and $\varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$

Where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓ_j denotes the effect of the j^{th} site (random effect), $r_{k(j)}$ denotes the effect of the k^{th} block within the j^{th} site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect), and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} site (random effect or residual). Notation $\sim iid\ N(0, \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a. Subscript *a* represents the corresponding source of variation.

The residual maximum likelihood estimation procedure was utilized to generate estimates of variance components and entry means across sites. The estimated means are known as empirical best linear unbiased estimators (hereafter referred to as LS-Means). The statistical comparison was conducted by testing for a difference in LS-Means between DP23211 maize and the control maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method (Kenward and Roger, 1997). A significant difference was identified if a P-value was < 0.05.

For each analyte, goodness-of-fit of the model was assessed in terms of meeting distributional assumptions of normally, independently distributed errors with homogeneous variance. Deviations from assumptions were addressed using an appropriate transformation or a

heterogeneous error variance structure. The statistical results for transformed data were back transformed to the original data scale for reporting purposes.

False Discovery Rate Adjustment

The false discovery rate (FDR) method (Benjamini and Hochberg, 1995; Westfall et al., 1999) was applied as a post-hoc procedure to control for false positive outcomes across all analytes analyzed using linear mixed models. A false positive outcome occurs if the difference in means between two entries is declared significant, when in fact the two means are not different. Since its introduction in the mid-1990s, the FDR approach has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science, and signal/image processing (*e.g.,* Pawitan et al., 2005; Spelman and Bovenhuis, 1998). In the FDR method, the false discovery rate is held at 5% across comparisons of multiple analytes via an adjustment to the P-value and is not inflated by the number of analytes in the comparison.

Interpretations of Statistical Results

For a given analyte, when a statistically significant difference (P-value from mixed model analysis < 0.05, or Fisher's exact test P-value < 0.05) was identified in the across-site analysis, the respective range of individual values from DP23211 maize was compared to a tolerance interval. Tolerance intervals are expected to contain at least 99% of the values for corresponding analytes of the conventional maize population with a 95% confidence level (Hong et al., 2014). The tolerance intervals were derived from Pioneer's proprietary accumulated data from non-GE maize lines, which were grown in commercial maize-growing regions in the United States, Canada, Chile, Brazil, and Argentina between 2003 and 2017. The combined data represent 144 unique non-GE commercial maize lines and 148 unique environments. The selected commercial maize lines represent the non-GE maize population with a history of safe use, and the selected environments (site and year combinations) represent maize growth under a wide range of environmental conditions (*i.e*. soil texture, temperature, precipitation, and irrigation) and maize maturity group zones.

If the range of DP23211 maize contained individual values outside the tolerance interval, it was then compared to the respective literature range obtained from published literature (Codex Alimentarius Commission, 2013; Cong et al., 2015; ILSI, 2016; Lundry et al., 2013; OECD, 2002; Watson, 1982). Literature ranges compliment tolerance intervals in that they are composed of non-proprietary data from additional non-GE commercial maize lines and growing environments, which are not included in Pioneer's proprietary database.

If the range of DP23211 maize contained individual values outside the literature range, it was then compared to the respective in-study reference range comprised of all individual values

across-sites from all non-GE reference maize lines grown in this study. In-study reference data ranges compliment tolerance intervals and literature ranges in that they provide additional context of natural variation specific to the current study.

In cases when a raw P-value indicated a significant difference but the FDR adjusted P-value was > 0.05, it was concluded that the difference was likely a false positive.

Appendix 11. Materials and Methods for Agronomic Performance Assessment

Evaluation of Germination and Viability of DP23211 Maize

Materials

The test system in this study was maize (*Zea mays* L.). The test substance consisted of event DP-Ø23211-2 contained within maize seed.

The control substance consisted of non-genetically engineered (non-GE) near-isoline maize seed (referred to as control maize), which did not contain event DP-Ø23211-2. Additionally, a total of six non-GE commercial maize lines (collectively referred to as reference maize) were included in the study as reference substances.

Methods

Experimental Design

Three separate germination tests (warm, cold, and diurnal) were conducted. For a given germination test, 400 seed from each of DP23211 maize, control maize, P0604 maize, P0760 maize, P0928 maize, P0993 maize, P1151 maize, and P1197 maize were evaluated. The seed from each maize line were arranged into eight individual replicates, with 50 seed per replicate.

Procedures employed to control bias in this study included random placement of each germination replicate within each container and uniform maintenance of environmental conditions across all replicates within each controlled environment.

Germination Tests

For a given germination test, each 50-seed replicate was placed between sheets of moist germination toweling and rolled up with a piece of wax paper. The rolls were vertically placed in storage racks.

For the warm germination test, the rolls were transferred to a 24-hour light controlled environment at a continuous setting of 25 °C and 95% relative humidity for 7 days. After 7 days, the number of normal and abnormal germinated seed and the number of hard, fresh, or dead ungerminated seed in each roll were counted.

For the cold germination test, the rolls were transferred to a 24-hour light controlled environment at a continuous setting of 10 °C and 95% relative humidity for 7 days, followed by 5 days at a continuous setting of 25 °C and 95% relative humidity. After 12 days, each replicate was evaluated and the number of normal and abnormal germinated seed and the number of hard, fresh, or dead ungerminated seed in each roll were counted.

For the diurnal germination test, the rolls were transferred to a 24-hour light controlled environment at a cyclical setting of 10 °C and 95% relative humidity for 16 hours followed by 25 °C and 95% relative humidity for 8 hours, repeated daily for 10 days; with the exception of one day where the rolls were transferred to the 24-hour light controlled environment 7.5 hours later than scheduled, resulting in exposure to a setting of 10 °C and 95% relative humidity for 8.5 hours and 25 °C and 95% relative humidity for 15.5 hours. After 10 days, the number of normal and abnormal germinated seed and the number of hard, fresh, or dead ungerminated seed in each roll were counted.

Seed Evaluation

Classification of Germinated and Ungerminated Seed

At the end of each germination test, replicates were evaluated and each seed was classified as either germinated (normal or abnormal) or ungerminated.

Germinated seed were considered viable. Ungerminated seed classified as dead were considered non-viable. If ungerminated seed classified as hard or fresh were identified, a tetrazolium chloride (TZ) test could have been conducted to assess viability; however, no hard or fresh seed were identified.

Statistical Methods

Statistical analyses of germination data were conducted to evaluate the germination rate of seed derived from DP23211 maize compared to the germination rate of seed derived from the control maize. Statistical analyses were conducted separately for each of the three germination tests (warm, cold, and diurnal) using SAS software (Version 9.4).

For a given germination test, Fisher's exact test was conducted to compare germination rates. A significant difference was established if the P-value < 0.05. SAS PROC FREQ was utilized to conduct Fisher's exact test.

Reported statistics for each germination test included descriptive statistics (total germination frequency, mean and range of germination rates for individual replicates, labeled as Frequency, Mean, and Range, respectively) for DP23211 maize and the control maize, and P-values for statistical comparisons between the two (labeled as P-value). For each germination test, the range of germination rates for individual replicates across reference maize lines (labeled as Reference Range) is provided.

Evaluation of Field Agronomic Performance

Materials

The test system in this study was maize (*Zea mays* L.). The test substance consisted of event DP-Ø23211-2 contained within maize seed.

The control line consisted of non-genetically engineered (non-GE) near-isoline maize seed (referred to as control maize), which did not contain event DP-Ø23211-2. Additionally, a total of 14 non-GE commercial maize lines (collectively referred to as reference maize) were included in the study.

Methods

Experimental Design

The field portion of this study was conducted during the 2018 growing season at 12 sites in commercial maize-growing regions of the United States (one site in Indiana, Minnesota, Nebraska, and Pennsylvania; two sites in Illinois and Texas; and three sites in Iowa) and Canada (one site in Ontario). A randomized complete block design with four blocks was utilized at each site. Each block included DP23211 maize, non-genetically engineered (non-GE) near-isoline control maize (referred to as control maize), and four of the following non-GE maize lines: P0604, 2R602, 35A52, P0760, BK5883, XL5939, P0928, P0993, XL5828, BK6076, XL6158, P1105, P1151, and P1197 maize.

Bias in this study was controlled by randomization of the entries within each block and uniform maintenance treatments across each plot area.

Planting

Each block contained DP23211 maize, non-GE control maize, and four reference maize lines planted in 6-row plots at a rate of 30 seeds per row. Each row was 20 ft (6.1 m) in length and 30 in. (76 cm) in width (except for site RG086TX1 where row width was 39 in.). Each block was separated by an alley of at least 3 ft. (0.9 m) in width, and each plot was bordered on either side by one row of maize.

Maintenance Product Applications

Maintenance products were uniformly applied to GE and non-GE control maize, as needed, at each site to minimize weed, insect, and disease pressure. Glufosinate-ammonium herbicides and insecticides containing *Bacillus thuringiensis* (*Bt*) were not used post emergence as maintenance

pesticides in this study. Details regarding maintenance product applications are presented inTable 142.

Herbicide Treatment

A nicosulfuron, diflufenzopyr, and dicamba herbicide treatment was applied to all control maize and reference maize plots, and to one of the two plots of DP23211 maize per block at the V4 growth stage (except for site RG086TX1 where the treatment was applied at the V5 growth stage). Maize growth stage descriptions are provided in Table 139. Details regarding herbicide treatments are provided in Table 142. A visual evaluation of the plants was completed 10-15 days after each treatment to confirm no unexpected herbicide injury was observed.

Table 144. Herbicide Treatments and Maintenance Products Used in DP23211 Maize Field Trials

Site	Date	Active Ingredient(s)	Commercial Product	Rate
RG086IA1	11/14/2017	N-P-K	Fertilizer	22-103-203 lb/A
	4/13/2018	Nitrogen	Fertilizer	200 lb/A
	5/8/2018	Mesotrione	Incinerate	3 fl oz/A
		S-metolachlor	Charger Max	1.67 pt/A
		Atrazine	Atrazine 90 DF	2.2 lb/A
		Glyphosate	Roundup WeatherMax	32 fl oz/A
RG086IA3	5/24/2018	Nitrogen	Fertilizer	92 lb/A
RG086IA5	4/28/2018	Nitrogen	Fertilizer	150 lb/A
		N-P-K	Fertilizer	11-52-150 lb/A
	5/19/2018	Acetochlor/Atrazine	Harness Xtra 5.6L	1 qt/A
RG086IL5	4/30/2018	Nitrogen	Fertilizer	180 lb/A
	5/14/2018	S-metolachlor/Mesotrione/ Atrazine/Bicyclopyrone	Acuron	80 fl oz/A
		Zeta-cypermethrin	Mustang Maxx	2.8 fl oz/A
RG086IL7	5/8/2018	Nitrogen	Fertilizer	184 lb/A
	5/16/2018	S-metolachlor/Atrazine/ Mesotrione	Lexar EZ	3 qt/A
		Lambda-cyhalothrin	Warrior II	1.3 oz/A
RG086IN2	4/26/2018	Nitrogen	Fertilizer	240 lb/A
	5/9/2018	S-metolachlor/Mesotrione/ Atrazine/Bicyclopyrone	Acuron	2.5 qt/A
	7/13/2018	Azoxystrobin/Propiconazole	Quilt Xcel	10.5 oz/A
		Lambda- cyhalothrin	Warrior	3.0 oz/A

Table 142. Herbicide Treatments and Maintenance Products Used in DP23211 Maize Field Trials (continued)

Site	Date	Active Ingredient(s)	Commercial Product	Rate
RG086MN1	4/28/2018	N-P-K	Fertilizer	0-40-120 lb/A
	4/29/2018	Nitrogen	Fertilizer	120 lb/A
	5/18/2018	Dimethenamid-P	Outlook	20 oz/A
		Atrazine	Atrazine 4L	3 pt/A
	5/31/2018	Tembotrione	Landis	3 oz/A
		Atrazine	Atrazine 4L	1 pt/A
RG086NE1	11/13/2017	N-P-K	Fertilizer	11-52-0 lb/A
	4/12/2018	Nitrogen	Fertilizer	32 lb/A
	5/18/2018	S-metolachlor/Atrazine/ Mesotrione/Bicyclopyrone	Acuron	2.5 qt/A
		Atrazine	Atrazine	1 qt/A
	7/25/2018[a]	Bifenthrin	Brigade	6 oz/A
		Lambda-cyhalothrin	Warrior	1.6 oz/A
		Benzovindiflupyr/ Azoxystrobin/Propiconazole	Trivapro	13.7 oz/A
RG086ON3B	5/9/2018	Nitrogen	Fertilizer	200 lb/A
	5/9/2018	N-P-K	Fertilizer	12.5-50-50 lb/A
	5/25/2018	S-metolachlor	Dual II Magnum	1.75 L/ha
		Mesotrione	Callisto	0.3 L/ha
		Atrazine	Aatrex	3.0 L/ha
RG086PA1	5/26/2018	Nitrogen	Fertilizer	92 lb/A
	5/29/2018	N-P-K	Fertilizer	45-33-51 lb/A
		Tefluthrin	Force 3G	5 oz per 1000 ft
		Mesotrione	Callisto	3.0 fl oz/A
		S- metolachlor	Dual II Magnum	1.5 pt/A
		Atrazine	Atrazine 4L	1.25 qt/A
		Glyphosate	Roundup PowerMax	1.5 qt/A

Table 142. Herbicide Treatments and Maintenance Products Used in DP23211 Maize Field Trials (continued)

Site	Date	Active Ingredient(s)	Commercial Product	Rate
RG086TX1	4/30/2018	N-P-K	Fertilizer	155-78-0 lb ai/A
	5/14/2018	Metolachlor	Medal	1.0 pt/A
		Atrazine	Atrazine 4L	1.5 pt/A
	6/11/2018	Zeta-cypermethrin	Mustang Maxx	4.0 fl oz/A
	6/25/2018[b]	Flubendiamide	Belt	3.0 fl oz/A
		Zeta-cypermethrin	Mustang Maxx	4.0 fl oz/A
		Azoxystrobin	Quadris	6.0 fl oz/A
	7/3/2018[c]	Chlorantraniliprole/Lambda-cyhalothrin	Besiege	10.0 fl oz/A
RG086TX7	5/18/2018	N-P-K	Fertilizer	25-30-0 lb/A
		S-metolachlor/Atrazine/ Mesotrione/Bicyclopyrone	Acuron	2.5 qt/A
	6/18/2018	Nitrogen	Fertilizer	35 lb/A
	6/28/2018	Atrazine	Atrazine	2 pt/A
	7/2/2018	Nitrogen	Fertilizer	120 lb/A

Note: Acre (A), active ingredient (ai), fluid oz (fl oz), foot (ft), hectare (ha), liter (L), ounce (oz), pint (pt), pound (lb), and quart (qt).

[a] Non-ionic surfactant was included in this application at a rate of 0.3 oz/A.

[b] The adjuvant Induce was added to this application at a rate of 0.25% v/v.

[c] The adjuvant Penetrator Plus was added to this application at a rate of 6.0 fl oz/A.

Pollination

To ensure grain purity, plants were self-pollinated by hand. Ear shoots in Rows 5 and 6 (and Rows 1 and 2 at site RG086ON3B) were covered prior to silk emergence and primary ears were pollinated.

Agronomic Characteristics Data Collection

The following characteristics were evaluated in Rows 1-4: early stand count, days to flowering, pollen viability, plant height, lodging, final stand count, and dropped ears. Days to maturity was evaluated in Rows 1 and/or 2 and yield, harvest grain moisture, and 100-kernel weight were evaluated in Rows 3 and 4.

Early Stand Count

The total number of emerged plants was determined between the V2 and V4 growth stages.

Days to Flowering

The date when approximately 50% of plants had begun shedding pollen was recorded. These dates were used in subsequent statistical analysis to calculate days to flowering.

Pollen Viability (Shape and Color at 0, 30, 60, and 120 Minutes)

When plants were actively shedding pollen, the percentage of non-viable pollen grains was assessed at four time points by recording the percentage of grains with collapsed walls and the percentage of grains with yellow color (Luna et al., 2001).

Plant Height

Plant height was measured in centimeters from the soil surface to the collar of the flag leaf (base of the tassel) for five individual plants at the R4 growth stage.

Days to Maturity

The date when the majority of the plants first reached physiological maturity was recorded.

Lodging

Lodging was evaluated at the R6 growth stage. Stalk lodging was recorded as the number of plants in each plot with stalks broken below the primary ear. Root lodging was recorded as the number of plants in each plot with stalks leaning approximately 45° or more. A combined lodging score was calculated from stalk and root lodging values.

Final Stand Count

The total number of remaining plants was recorded at the R6 growth stage.

Dropped Ears

The number of dropped ears (ears lying on the ground within each plot) was recorded at the R6 growth stage.

Yield

The grain from two rows in each plot was harvested at the R6 growth stage. The weight of the grain was recorded in pounds at all sites. Grain weight values from all sites were adjusted to a standardized moisture content and used to calculate yield during subsequent statistical analysis.

Harvest Grain Moisture

The moisture content (%) of harvested grain at the R6 growth stage was recorded.

100-Kernel Weight

The total weight (g) of 100 kernels sampled from the pooled grain harvested from two rows in each plot was determined. 100-kernel weight values were adjusted to a standardized moisture content.

The following exceptions occurred during agronomic characteristics data collection: At RG086TX7, the following characteristics were not available from one block due to herbicide drift: days to flowering, pollen viability (shape and color at 0, 30, 60, and 120 minutes), plant height, days to maturity, lodging, final stand count, dropped ears, yield, harvest grain moisture, and 100-kernel weight. At RG086ON3B, plant height data were collected at the R5 growth stage instead of the R4 growth stage. At RG086TX1, 100-kernel weight and grain moisture were collected from 5 ears in rows 1 and/or 2 on a different date than yield.

Statistical Methods

Statistical analyses were conducted to evaluate and compare agronomic characteristics of DP23211 maize and the control maize.

Processing of Data

Early Stand Count and Final Stand Count

For early stand count and final stand count data, the recorded count value was divided by count area to calculate the number of plants per m^2.

Days to Flowering and Days to Maturity

For days to flowering data, the number of days was calculated from the recorded planting date to the recorded flowering date. For days to maturity data, the number of days was calculated from the recorded planting date to the recorded maturity date.

Plant Height

For plant height data, the recorded values for five individual plants were used to calculate the plot average.

Lodging

For lodging data, the numbers of root-lodged plants and stalk-lodged plants were summed and then divided by the final stand count to convert to a percentage basis.

Yield

Yield was determined based on the weight of grain collected at typical harvest maturity as follows:

Grain weight was adjusted to 0% moisture content (Grain dry weight):

Grain dry weight (lb) = Grain fresh weight (lb) $\times$ (1 - % actual moisture)

Grain dry weight was then adjusted to 15.5% moisture content:

Grain weight at 15.5% moisture (lb) = Grain dry weight (lb) / (1 – 15.5% moisture)

Grain weight at 15.5% moisture was then converted to a yield in bushels per acre (bu/A):

$$\text{Yield (bu/A at 15.5\% moisture)} = \frac{\text{(Grain weight (lb) at 15.5\% moisture)} \times (43{,}560\ \text{ft}^2/\text{A})}{(\text{plot area } (\text{ft}^2)) \times (56\ \text{lb/bu})}$$

Plot area was calculated by first converting unit of measurement to feet and then using the following formula:

plot area (ft^2) = row length (ft) $\times$ row width (ft) $\times$ number of rows.

100-Kernel Weight

100-kernel weight for each plot was determined as follows:

Weight of 100 kernels was adjusted to 0% moisture content (100-kernel dry weight):

100-kernel dry weight (g) = 100-kernel fresh weight (g) × (1 - % actual moisture)

100-kernel dry weight was then adjusted to 15.5% moisture content:

100-kernel weight at 15.5% moisture (g) = 100-kernel dry weight (g) / (1 – 15.5% moisture)

Selection of Statistical Method

The following rules were implemented for each agronomic characteristic:

If < 50% of sites had uniform data values for either DP23211 maize or the control maize, and < 50% of all data across sites for each entry were at a uniform value, then an across-site mixed model analysis would be conducted.

If ≥ 50% of sites had uniform data values for either DP23211 maize or the control maize, and ≥ 50% of sites had uniform data values across both maize lines, then statistical analyses would not be performed.

If the criteria described above were not met, then an across-site analysis using the generalized Cochran-Mantel-Haenszel (CMH) test would be conducted. Individual-site analyses would not be performed.

Across-Site Analysis

Mixed Model Analysis

For a given agronomic characteristic, data were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ Model 1

$\ell_j \sim iid\ N(0, \sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0, \sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Ent\times Site})$, and $\varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$,

where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓ_j denotes the effect of the j^{th} site (random effect), $r_{k(j)}$ denotes the effect of the k^{th} block within the j^{th} site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect), and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} site (random effect or residual). Notation $\sim iid\ N(0, \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a. Subscript *a* represents the corresponding source of variation.

The residual maximum likelihood estimation procedure was utilized to generate estimates of variance components and entry means across sites. The estimated means are known as empirical best linear unbiased estimators (hereafter referred to as LS-Means). The statistical comparison was conducted by testing for a difference in LS-Means between DP23211 maize or the control

maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method (Kenward and Roger, 2009). A significant difference was identified if a P-value was < 0.05.

For each agronomic characteristic, goodness-of-fit of the model was assessed in terms of meeting distributional assumptions of normally, independently distributed errors with homogeneous variance. Deviations from assumptions were addressed using an appropriate transformation or allowing for heterogeneous error variance among sites. The statistical results for transformed data were back-transformed to the original data scale for reporting purposes.

Generalized CMH Test

The generalized CMH test is more appropriate in the instance where the normality assumption of mixed model analysis cannot be achieved for discrete data. The test was developed specifically for stratified nominal-by-ordinal contingency tables (Agresti, 2002; Koch et al., 1990). It compares entries (a nominal variable) based on their values (recorded on an ordinal scale) while controlling for location (the stratifying variable). Due to the data values being used as the scores in the generalized CMH test, the test's P-value can be directly interpreted as testing for the difference between the arithmetic means of two entries. A significant difference was identified if a P-value was < 0.05.

False Discovery Rate Adjustment

The false discovery rate (FDR) method (Benjamini and Hochberg, 1995; Westfall et al., 1999) was used to control for false positive outcomes across all agronomic characteristics analyzed using linear mixed models or generalized CMH tests. A false positive outcome occurs if the difference in means between two entries is declared significant, when in fact the two means are not different. Since the introduction of the FDR approach in the mid-1990s, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science, and signal/image processing (*e.g.*, Pawitan et al., 2005; Spelman and Bovenhuis, 1998). In the FDR method, the false discovery rate is held at 5% across comparisons of multiple agronomic characteristics via an adjustment to the p-value and is not inflated by the number of agronomic characteristics in the comparison. The FDR adjustment of raw P-values was conducted separately for the across-site analysis and each of the individual-site analyses.

Statistical Software and Procedures

Statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA). SAS PROC MIXED was utilized to fit Models 1 and 2, and to provide LS-Means, 95% confidence intervals, and statistical comparisons. SAS PROC FREQ was used to perform the

generalized CMH test. SAS PROC MULTTEST was utilized to provide FDR adjusted P-values. All other data processing was generated by Base SAS.

Interpretation of Statistical Results

For a given agronomic characteristic, when a statistically significant difference (P-value < 0.05) was identified in the across-site analysis, the respective range of individual values from DP23211 maize was compared to the in-study reference range comprised of all individual values across-sites from all non-GM reference maize lines grown in this study. In cases when a raw P-value indicated a significant difference but the FDR adjusted P-value was >0.05, it was concluded that the difference was likely a false positive. In addition, for agronomic characteristics exhibiting a statistically significant difference (P-value < 0.05) in the across-site analysis, the results for individual sites were evaluated.

Appendix 12. Materials and Methods for Efficacy Evaluation

Materials

The test system was hybrid maize (*Zea mays* L.). The test substance consisted of maize plants containing event DP-Ø23211-2. The control line consisted of non-genetically modified (non-GM) near-isoline hybrid maize plants (referred to as control maize), which did not contain event DP-Ø23211-2.

Experimental Design

Field testing was conducted in 2018 at 14 locations in commercial maize-growing regions of North America to evaluate efficacy of DP23211 maize against CRW. Each site included DP23211 maize and a negative control maize in single-row plots, utilizing a randomized complete block design with three replications. Plots were 10 feet (3.0 m) in length.

Trait Confirmation

Prior to planting, 42 kernels from each seed lot were characterized using PCR to confirm the presence of the DP23211 event.

Infestation

A 4-foot section of each row was manually infested when plants reached V2-V4 growth stage with non-diapausing WCR eggs. Eggs were infested at a targeted infestation rate of approximately 750 eggs/plant or 1500 eggs/plant, depending on the location. Eggs were injected into the soil approximately 4 inches (10 cm) deep and approximately 2-3 inches (5-8 cm) on both sides of each plant.

Root Injury Evaluation

Roots were visually evaluated between 56 and 78 days after planting for the presence of injury from larval feeding. Plants were at approximately the VT to R2 growth stages at the time of evaluation. Two maize plants from the infested region in each plot were tagged, manually dug from the ground, washed clean of soil with pressurized water, and were visually evaluated for larval feeding contained on each root. The corn rootworm node-injury score (CRWNIS) was recorded for each plant using the Iowa State 0-3 node-injury scale described in Oleson et al. (2005). No CRWNIS ratings were collected at five of the 14 locations due to a low nodal injury score (nodal injury below 0.75) on negative control roots.

Statistical Analysis

Statistical analyses were conducted to evaluate and compare CRWNIS data for DP23211 maize and the control maize across nine locations.

Data were analyzed using the following linear mixed model:

$$Y_{ijgmks} = \mu + L_i + \underline{(L\times R)_{ij}} + \underline{G_g} + \underline{(L\times G\ )_{ig}} + \underline{(L\times R\times G\ )_{iJg}} + P_m + \underline{(G\times P\ )_{gm}} + \underline{(L\times P)_{im}} + \underline{(L\times G\times P\ )_{igm}} + \underline{(K/L\ )_{ik}} + \underline{(\varepsilon/L)_{ijgmks}}$$

Where data for root injury (Y_{ijgmks}) of location $(L)_i$, replication $(R)_j$, background $(G)_g$, event $(P)_m$, plot $(K)_k$ and plant (s), were modeled as a function of an overall mean μ, factors for location, location by replication, background, location by background, location by replication by background, event, background by event, location by event, location by background by event, plot within each location $(K/L\ )_{ik}$ and a residual within each location $(\varepsilon/L)_{ijgmks}$.

Location and event were treated as fixed effect, and all the other effects were treated as independent normally distributed random variables with means of zero. *T*-tests using standard errors from the model were conducted to compare treatment effects. A difference was considered statistically significant if the *P*-value of the difference was less than 0.05. All statistical data analysis and comparisons were conducted using ASReml 3.0 (VSN International, Hemel Hempstead, UK, 2009).

Appendix 13. Materials and Methods for Field Insect and Disease Observations

Experiment A - 2018 Field Trial Biotic and Abiotic Stressor Measurement

A field trial was conducted during the 2018 growing season at 12 sites in maize-growing regions of the United States (one site in Indiana, Minnesota, Nebraska, and Pennsylvania; two sites in Illinois and Texas; and three sites in Iowa) and Canada (one site in Ontario). Biotic and abiotic observations were collected from the same field plots from which other agronomic data (Appendix 11. Materials and Methods for Agronomic Performance Assessment), and composition (Appendix 10. Materials and Methods for Nutrient Composition Assessment) and expression samples (Appendix 6. Methods for Determination of DvSSJ1 dsRNA and IPD072Aa, PAT, and PMI Protein Concentrations) were collected. Plot information, including size of plots, is found in Appendix 11. Each plot contained 6 rows and was bordered on either side by one row of maize planted with seed of the same lot as the 6-row plot. Each row was 20 ft. (6.1 m) in length and 30 in. (76 cm) in width (except for site RG086TX1 where row width was 39 in.). Each block was separated by an alley of at least 3 ft. (0.9 m) in width. The entire field trial site was surrounded with a minimum of four external border rows and a minimum of 10 ft. (3 m) of bare ground buffer area surrounding the external border rows. The intent of the biotic and abiotic stress observation collection was not to show efficacy of the trait, and not to conduct a survey of non-target organisms, but to observe if there may be potential differential responses in biotic and abiotic stressors between DP23211 maize and control and/or reference lines within the same site.

Each site utilized a randomized complete block design with four blocks, and each block included DP23211 maize, non-GE near isoline control maize, and four of the following non-GM reference maize lines: P0604, 2R602, 35A52, P0760, BK5883, XL5939, P0928, P0993, XL5828, BK6076, XL6158, P1105, P1151, and P1197 maize.

For each site, entry randomization was conducted prior to planting using a macro developed in Microsoft Excel that utilized the program's RAND function. The four reference lines at each site were selected from a set of 14 non-GM commercial reference lines, listed above. When distributing reference lines to sites, the CRM zone of each site as well as the CRM of each reference line, was considered. The CRM of the reference lines ranged from 106-111.

Bias in this portion of the study was controlled by randomization of maize entries within each block and uniform maintenance treatments across each plot area.

A nicosulfuron, diflufenzopyr, and dicamba herbicide treatment was applied to all control maize and reference maize plots, and to the two plots of DP23211 maize per block at the V4 growth stage, with the exception of site RG086TX1 where the treatment was applied at the V5 growth stage. A visual evaluation of the plants was completed 10-15 days after each treatment. Details regarding herbicide treatments and evaluation dates are provided in Table 143. The herbicide

treatments were applied across all sites and are appropriate for each of the specific sites. Each treatment provides control of common weed species and were sprayed at commercially labeled rates and crop growth stages.

Table 145. Field Phase Dates

Agronomic Characteristic	Site					
	RG086IA1	RG086IA3	RG086IA5	RG086IL5	RG086IL7	RG086IN2
Planting	5/8/2018	5/27/2018	5/18/2018	5/10/2018	5/15/2018	5/25/2018
Herbicide Application	5/31/2018	6/22/2018	6/7/2018	5/28/2018	6/4/2018	6/18/2018
Herbicide Evaluation	6/11/2018	7/6/2018	6/21/2018	6/11/2018	6/14/2018	7/2/2018

Table 143 Field Phase Dates (continued)

Agronomic Characteristic	Site					
	RG086MN1	RG086NE1	RG086ON3B	RG086PA1	RG086TX1	RG086TX7
Planting	5/16/2018	5/17/2018	5/24/2018	5/29/2018	5/11/2018	5/17/2018
Herbicide Application	6/5/2018	6/8/2018	6/15/2018	6/21/2018	6/5/2018	6/8/2018
Herbicide Evaluation	6/15/2018	6/21/2018	6/30/2018	7/2/2018	6/20/2018	6/22/2018

At a given site, maintenance products were uniformly applied, as needed, to all plots to minimize weed, insect, and disease pressure. Glufosinate-ammonium herbicides and insecticides containing *Bacillus thuringiensis* (*Bt*) were not used post emergence as maintenance applications in this study. Details regarding maintenance product applications are provided inTable 144.

Table 146. Herbicide Treatments and Maintenance Product Applications

Site	Date	Active Ingredient(s)	Commercial Product	Rate
RG086IA1	11/14/2017	N-P-K	Fertilizer	22-103-203 lb/A
	4/13/2018	Nitrogen	Fertilizer	200 lb/A
	5/8/2018	Mesotrione	Incinerate	3 fl oz/A
		S-metolachlor	Charger Max	1.67 pt/A
		Atrazine	Atrazine 90 DF	2.2 lb/A
		Glyphosate	Roundup WeatherMax	32 fl oz/A
RG086IA3	5/24/2018	Nitrogen	Fertilizer	92 lb/A
RG086IA5	4/28/2018	Nitrogen	Fertilizer	150 lb/A
		N-P-K	Fertilizer	11-52-150 lb/A
	5/19/2018	Acetochlor/Atrazine	Harness Xtra 5.6L	1 qt/A
RG086IL5	4/30/2018	Nitrogen	Fertilizer	180 lb/A
	5/14/2018	S-metolachlor/Mesotrione/Atrazine/Bicyclopyrone	Acuron	80 fl oz/A
		Zeta-cypermethrin	Mustang Maxx	2.8 fl oz/A
RG086IL7	5/8/2018	Nitrogen	Fertilizer	184 lb/A
	5/16/2018	S-metolachlor/Atrazine/Mesotrione	Lexar EZ	3 qt/A
		Lambda-cyhalothrin	Warrior II	1.3 oz/A
RG086IN2	4/26/2018	Nitrogen	Fertilizer	240 lb/A
	5/9/2018	S-metolachlor/Mesotrione/Atrazine/Bicyclopyrone	Acuron	2.5 qt/A
	7/13/2018	Azoxystrobin/Propiconazole	Quilt Xcel	10.5 oz/A
		Lambda- cyhalothrin	Warrior	3.0 oz/A
RG086MN1	4/28/2018	N-P-K	Fertilizer	0-40-120 lb/A
	4/29/2018	Nitrogen	Fertilizer	120 lb/A
	5/18/2018	Dimethenamid-P	Outlook	20 oz/A
		Atrazine	Atrazine 4L	3 pt/A
	5/31/2018	Tembotrione	Landis	3 oz/A
		Atrazine	Atrazine 4L	1 pt/A
RG086NE1	11/13/2017	N-P-K	Fertilizer	11-52-0 lb/A
	4/12/2018	Nitrogen	Fertilizer	32 lb/A
	5/18/2018	S-metolachlor/Atrazine/ Mesotrione/Bicyclopyrone	Acuron	2.5 qt/A
		Atrazine	Atrazine	1 qt/A
	7/25/2018[a]	Bifenthrin	Brigade	6 oz/A
		Lambda-cyhalothrin	Warrior	1.6 oz/A
		Benzovindiflupyr/Azoxystrobin/Propiconazole	Trivapro	13.7 oz/A

Table 144 Herbicide Treatments and Maintenance Product Applications (continued)

Site	Date	Active Ingredient(s)	Commercial Product	Rate
RG086ON3B	5/9/2018	Nitrogen	Fertilizer	200 lb/A
	5/9/2018	N-P-K	Fertilizer	12.5-50-50 lb/A
	5/25/2018	S-metolachlor	Dual II Magnum	1.75 L/ha
		Mesotrione	Callisto	0.3 L/ha
		Atrazine	Aatrex	3.0 L/ha
RG086PA1	5/26/2018	Nitrogen	Fertilizer	92 lb/A
	5/29/2018	N-P-K	Fertilizer	45-33-51 lb/A
		Tefluthrin	Force 3G	5 oz per 1000 ft
		Mesotrione	Callisto	3.0 fl oz/A
		S- metolachlor	Dual II Magnum	1.5 pt/A
		Atrazine	Atrazine 4L	1.25 qt/A
		Glyphosate	Roundup PowerMax	1.5 qt/A
RG086TX1	4/30/2018	N-P-K	Fertilizer	155-78-0 lb ai/A
	5/14/2018	Metolachlor	Medal	1.0 pt/A
		Atrazine	Atrazine 4L	1.5 pt/A
	6/11/2018	Zeta-cypermethrin	Mustang Maxx	4.0 fl oz/A
	6/25/2018[b]	Flubendiamide	Belt	3.0 fl oz/A
		Zeta-cypermethrin	Mustang Maxx	4.0 fl oz/A
		Azoxystrobin	Quadris	6.0 fl oz/A
	7/3/2018[c]	Chlorantraniliprole/Lambda-cyhalothrin	Besiege	10.0 fl oz/A
RG086TX7	5/18/2018	N-P-K	Fertilizer	25-30-0 lb/A
		S-etolachlor/Atrazine/Mesotrione/Bicyclopyrone	Acuron	2.5 qt/A
	6/18/2018	Nitrogen	Fertilizer	35 lb/A
	6/28/2018	Atrazine	Atrazine	2 pt/A
	7/2/2018	Nitrogen	Fertilizer	120 lb/A

Biotic and abiotic observations were taken from Rows 1-4 of each plot. Each plot was evaluated for four observation periods: early vegetative (V2-V5), late vegetative (V7-V9), early reproductive (R1-R2), and late reproductive (R3-R6) growth stages. Evaluation dates for biotic and abiotic characteristic are provided in Tables 149-160. Insect damage incidence, plant pathogen incidence, and abiotic stress were evaluated by recording the severity of plant tissue damage caused by each of three insects predominant to the local area, three pathogens predominant to the local area, and three abiotic stressors, respectively. The following ratings were used to evaluate plant damage (Table 145): “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); “moderate” indicates intermediate symptoms between slight and severe; and “severe” indicates symptoms damaging to plant development (*e.g.*, stunting or death). Maize insect stressors,

pathogen stressors, and abiotic stressors and observation periods are presented in Table 146, Table 147, and Table 148.

Table 147. Severity of Plant Tissue Rating Scale

Unit of Measure	Rating Codes	Severity of Plant Damage
None	NO	No damage/symptoms observed
Slight	SL	Symptoms not damaging to plant development, e.g., minor feeding or minor lesions (mitigation not required)
Moderate	MD	Intermediate between slight and severe (mitigation likely required)
Severe	SV	Symptoms damaging to plant development, e.g., stunting or death (mitigation not likely to be effective)

Table 148 Maize Insect Stressors

Insect Name	Insect Codes	Feeding Mode	Observation Period			
			V2-V5	V7-V9	R1-R2	R3-R6
Aphids	AP	Sap sucker	X	X	X	X
Armyworms	AM	Foliage chewer	X	X	X	
Armyworms, beet	BAM	Foliage chewer	X	X	X	
Armyworms, fall	FAM	Foliage chewer	X	X	X	X
Billbugs (weevils)	BB	Foliage chewer	X	X		
Corn earworm	CEW	Foliage chewer and grain feeder		X	X	X
Cutworms, black	BCW	Foliage chewer and stem feeder	X	X		
Cutworms, Western bean	WBC	Foliage chewer and grain feeder	X	X	X	X
European corn borer	ECB	Stem borer	X	X	X	X
Flea beetles	FB	Foliage chewer	X	X	X	
Grape colaspis	GC	Foliage chewer	X	X	X	
Grasshoppers	GH	Foliage chewer	X	X	X	X
Japanese beetles	JP	Foliage chewer and silk feeder		X	X	
Leafhoppers	LH	Sap sucker	X	X	X	
Rootworms (adults)	RW	Foliage chewer and silk feeder		X	X	X
Sap beetles	SB	Grain feeder		X	X	X
Southwestern corn borer	SCB	Stem borer	X	X	X	X
Spider mites	SM	Sap sucker		X	X	X
Stink bugs	SB	Sap sucker and grain feeder	X		X	
Sugarcane borer	SC	Stem borer	X	X	X	X
Thrips	TH	Sap sucker	X	X		
Other	*	Foliage chewer	X	X	X	X
Other	*	Grain feeder	X	X	X	X
Other	*	Sap sucker	X	X	X	X
Other	*	Stem borer	X	X	X	X

*"Other" stressors as noted during field observation period

Table 149. Maize Pathogen Stressors

Pathogen Name	Pathogen Codes	Infection Type	Observation Period			
			V2-V5	V7-V9	R1-R2	R3-R6
Anthracnose	AN	Foliar	X	X	X	X
Bacterial stalk rot of maize	BSR	Stalk		X	X	X
Corn stunt	CS	Whole Plant	X	X		
Crazy top	CT	Foliar			X	
Downy mildew	DM	Foliar		X	X	
Ear rot (*e.g.*, Diplodia, Fusarium)	ER	Ear			X	X
Eyespot	ES	Foliar	X	X	X	
Goss' bacterial wilt	GW	Foliar	X	X	X	X
Grey leaf spot of maize	GLS	Foliar	X	X	X	X
Leaf blight of maize (Northern)	NLB	Foliar	X	X	X	
Leaf blight of maize (Southern)	SLB	Foliar	X	X	X	
Leaf spot (Northern)	NLS	Foliar	X	X	X	
Maize dwarf mosaic virus	MDMV	Foliar	X	X		
Maize rough dwarf virus	MRDV	Foliar			X	X
Rust: Maize (common)	RSC	Foliar		X	X	X
Rust: Maize (Southern)	RSS	Foliar		X	X	X
Smut (common)	SMT	Ear			X	X
Stalk rot (*e.g.*, Pythium, Fusarium)	SR	Stalk		X	X	
Stewart's wilt	SW	Foliar	X	X	X	
Wheat streak mosaic virus	WSMV	Foliar	X	X		
Other	*	Foliar	X	X	X	X
Other	*	Stalk		X	X	X
Other	*	Root	X	X	X	X
Other	*	Ear	X	X	X	X

*"Other" stressors as noted during field observation period

Table 150 Maize Abiotic Stressors

Abiotic Stressor	Abiotic Stressor Codes	Observation Period			
		V2-V5	V7-V9	R1-R2	R3-R6
Cold stress (3 consecutive days ≤40°F)	CD	X	X	X	X
Drought	DR	X	X	X	X
Frost	FR	X			X
Hail	HL	X	X	X	X
Heat stress (3 consecutive days ≥95°F)	HS	X	X	X	X
Mineral toxicity	MT	X	X	X	X
Nutrient deficiency	ND	X	X	X	X
Soil compaction	SCP	X	X	X	X
Soil crusting	SCR	X			
Sun scald	SS	X	X	X	X
Waterlogging (excessive rain, wet soil, flooding)	WL	X	X	X	X
Wind damage (with plants blown over)	WD	X	X	X	X
Maintenance and non-target pesticide injury	MPI	X	X	X	X
Other	*	X	X	X	X

*"Other" stressors as noted during field observation period

At RG086NE1, V7-V9 biotic and abiotic observations were not collected due to a collection error. At RG086ON3B, an R3-R6 smut rating was not available from one plot of 2R602 due to a collection error. At RG086TX7, V7-V9, R1-R2, and R3-R6 data were not available from one block due to herbicide drift.

In Experiment A, management practices including tillage, nutrient management, pest management, and irrigation varied at each site based on specific needs and appropriate crop management practices for the site. In general, Principal Investigators follow IPM methods to evaluate plot health throughout the growing season, identify pests, and manage damage well before economic thresholds are met by applying specific pesticides. At a given site, maintenance products were uniformly applied, as determined by the Principal Investigator, to all plots to minimize weed, insect, and disease pressure (Table 144). Principal Investigators in this study followed protocol and GLP practices and have documented agricultural research training and experience. Principal Investigators used actual incidence in the local area, as well as historical experience to pre-determine which three disease, insect and abiotic stressors to record at each observation period.

The results for the biotic and abiotic observations are provided in Tables 149-160.

Table 151. Biotic and Abiotic Observations Across Blocks at Site RG086IA1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	XL5939 Maize	P0993 Maize	BK6076 Maize	XL6158 Maize
V2-V5 Growth Stages (Evaluated 5/30/2018)							
Insect Damage	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	ECB	None-Slight	None	None	None-Slight	None	None
Pathogen Stressor	CS	None	None	None	None	None	None
	ES	None	None	None	None	None	None
	NLS	None	None	None	None	None	None
Abiotic Stressor	HS	None	None	None	None	None	None
	SS	None	None-Slight	None	None-Slight	None-Slight	None-Slight
	WD	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/12/2018)							
Insect Damage	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	SB	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	NLB	None	None	None	None	None-Slight	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	SS	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 7/13/2018)							
Insect Damage	AP	None	None	None	None	None	None
	FB	None	None	None	None	None	None
	SB	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	GLS	None-Slight	None	None-Slight	None-Slight	None-Slight	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	SS	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None	None	None	None
R3-R6 Growth Stages (Evaluated 7/31/2018)							
Insect Damage	CEW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	GLS	Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight
	RSC	None-Slight	None	None	None-Slight	None-Slight	None-Slight
	SMT	None	None-Slight	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	MPI	None	None	None	None	None	None
	WD	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of aphids (AP), black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), flea beetles (FB), rootworm (RW), and stink bugs (SB). Pathogen stressors consisted of corn stunt (CS), eyespot (ES), grey leaf spot (GLS), northern leaf blight (NLB), northern leaf spot (NLS), common maize rust (RSC), and smut (SMT). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), sun scald (SS), wind damage (WD), and maintenance and non-target pesticide injury (MPI).

Table 152. Biotic and Abiotic Observations Across Blocks at Site RG086IA3

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	P0604 Maize	2R602 Maize	35A52 Maize	P0760 Maize
V2-V5 Growth Stages (Evaluated 6/14/2018)							
Insect Damage	BCW	Slight	Slight	Slight	Slight	Slight	Slight
	ECB	Slight	Slight	Slight	Slight	Slight	Slight
	SB	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
Abiotic Stressor	HS	None	None	None	None	None	None
	ND	Slight	Slight	Slight	Slight	Slight	Slight
	WL	Slight	Slight	Slight	Slight	Slight	Slight
V7-V9 Growth Stages (Evaluated 7/6/2018)							
Insect Damage	CEW	Slight	Slight	Slight	Slight	Slight	Slight
	ECB	Slight	Slight	Slight	Slight	Slight	Slight
	RW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
Abiotic Stressor	HS	None	None	None	None	None	None
	ND	Slight	Slight	Slight	Slight	Slight	Slight
	WL	Slight	Slight	Slight	Slight	Slight	Slight
R1-R2 Growth Stages (Evaluated 7/25/2018)							
Insect Damage	ECB	Slight	Slight	Slight	Slight	Slight	Slight
	RW	Slight	Slight	Slight	Slight	Slight	Slight
	SB	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	HS	Slight	Slight	Slight	Slight	Slight	Slight
	ND	Slight	Slight	Slight	Slight	Slight	Slight
	WL	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 9/30/2018)							
Insect Damage	ECB	Slight	Slight	Slight	Slight	Slight	Slight
	RW	Slight	Slight	Slight	Slight-Moderate	Slight	Slight
	SM	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	AN	Slight	Slight	Slight	Slight	Slight	Slight
	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	GW	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	ND	Slight	Slight	Slight	Slight	Slight	Slight
	WD	Slight	Slight	Slight	Slight	Slight	Slight
	WL	Slight	Slight	Slight	Slight	Slight	Slight

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of, black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), rootworm (RW), stink bugs (SB), and spider mites (SM). Pathogen stressors consisted of anthracnose (AN), Goss' bacterial wilt (GW), grey leaf spot (GLS), northern leaf blight (NLB), and common maize rust (RSC). Abiotic stressors consisted of heat stress (HS), nutrient deficiency (ND), wind damage (WD), and waterlogging (WL).

Table 153. Biotic and Abiotic Observations Across Blocks at Site RG086IA5

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	BK5883 Maize	XL5939 Maize	P0928 Maize	XL5828 Maize
V2-V5 Growth Stages (Evaluated 6/2/2018)							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	WBC	None	None	None	None	None	None
Pathogen Stressor	AN	Slight	Slight	Slight	Slight	Slight	Slight
	GW	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	SCP	None	None	None	None	None	None
	SCR	Slight	Slight-Moderate	Slight	Slight	Slight	Slight
	WL	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/21/2018)							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
Pathogen Stressor	AN	Slight	Slight	Slight	Slight	Slight	Slight
	ES	Slight	Slight	Slight	Slight	Slight	Slight
	SW	None	None	None	None	None	None
Abiotic Stressor	HL	Slight	Slight	Slight	Slight	Slight	Slight
	WD	Slight	Slight-Moderate	Slight	Slight	Slight	Slight
	WL	Slight	Slight	Slight	Slight	Slight	Slight
R1-R2 Growth Stages (Evaluated 7/26/2018)							
Insect Damage	AP	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	JP	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight-Moderate	Slight	Slight	Slight-Moderate
	NLB	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	HL	Slight	Slight	Slight	Slight	Slight	Slight
	HS	None	None	None	None	None	None
	WD	Slight-Moderate	Slight	Moderate	Slight-Moderate	Moderate-Severe	Slight
R3-R6 Growth Stages (Evaluated 9/4/2018)							
Insect Damage	CEW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
Pathogen Stressor	GLS	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
	GW	None	None	None	None	None	None
	SMT	None	None	None	None	None	None
Abiotic Stressor	FR	None	None	None	None	None	None
	WD	Slight	Slight	Slight	Slight	Slight-Moderate	Slight
	WL	Slight	Slight	Slight	Slight	Slight	Slight

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), grasshoppers (GH), Japanese beetles (JP), and western bean cutworms (WBC). Pathogen stressors consisted of anthracnose (AN), eyespot (ES), grey leaf spot (GLS), Goss' bacterial wilt (GW), northern leaf blight (NLB), common maize rust (RSC), smut (SMT), and Stewart's wilt (SW). Abiotic stressors consisted of frost (FR), hail (HL), heat stress (HS), soil compaction (SCP), soil crusting (SCR), wind damage (WD), and waterlogging (WL).

Table 154. Biotic and Abiotic Observations Across Blocks at Site RG086IL5

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	P0928 Maize	XL5828 Maize	XL6158 Maize	P1197 Maize
V2-V5 Growth Stages (Evaluated 6/1/2018)							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	FB	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	MDMV	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/14/2018)							
Insect Damage	AM	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
Pathogen Stressor	BSR	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	ND	None	None-Slight	None-Slight	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None-Slight	None-Slight	None	None	None
R1-R2 Growth Stages (Evaluated 7/12/2018)							
Insect Damage	AM	None	None	None	None	None	None
	JP	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	SCP	Slight	Slight	Slight	Slight	Slight	Slight
	WD	None	None	None	None	None	None
	WL	None	None-Slight	Slight	None-Slight	None-Slight	None
R3-R6 Growth Stages (Evaluated 8/22/2018)							
Insect Damage	CEW	None-Slight	Slight	None-Slight	None-Slight	None-Slight	None-Slight
	ECB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
Pathogen Stressor	ER	None	None	None	None-Slight	None	None
	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	SR	None	None	None-Slight	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None-Slight	None-Moderate	Slight-Moderate	None-Moderate
	WL	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), flea beetles (FB), grasshoppers (GH), Japanese beetles (JP), and rootworm (RW). Pathogen stressors consisted of anthracnose (AN), bacterial stalk rot of maize (BSR), ear rot (ER), grey leaf spot (GLS), maize dwarf mosaic virus (MDMV), northern leaf blight (NLB), common maize rust (RSC), stalk rot (SR), and Stewart's wilt (SW). Abiotic stressors consisted of drought (DR), nutrient deficiency (ND), soil compaction (SCP), wind damage (WD), and waterlogging (WL).

Table 155. Biotic and Abiotic Observations Across Blocks at Site RG086IL7

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	P0993 Maize	XL5828 Maize	BK6076 Maize	P1151 Maize
V2-V5 Growth Stages (Evaluated 6/6/2018)							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	FB	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	CS	None	None	None	None	None	None
	ES	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HL	None	None	None	None	None	None
	WD	Slight	Slight	Slight	Slight	Slight	Slight
V7-V9 Growth Stages (Evaluated 6/15/2018)							
Insect Damage	AP	Slight	Slight	Slight	Slight	Slight	Slight
	FB	Slight	Slight	Slight	Slight	Slight	Slight
	JP	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	SCP	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 7/11/2018)							
Insect Damage	FAW	None	None	None-Slight	None	None	None
	FB	Slight	Slight	Slight	Slight	Slight	Slight
	GH	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	RSC	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	SCP	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 8/24/2018)							
Insect Damage	CEW	None	None	None	None	None	None
	FAW	None	None	None	None	None	None
	GH	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	RSC	None	None	None	None	None	None
	SMT	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), black cutworms (BCW), corn ear worm (CEW), fall armyworms (FAW), flea beetles (FB), grasshoppers (GH), and Japanese beetles (JP). Pathogen stressors consisted of corn stunt (CS), eyespot (ES), grey leaf spot (GLS), northern leaf blight (NLB), common maize rust (RSC), smut (SMT), and Stewart's wilt (SW). Abiotic stressors consisted of drought (DR), hail (HL), heat stress (HS), nutrient deficiency (ND), soil compaction (SCP), wind damage (WD), and waterlogging (WL).

Table 156. Biotic and Abiotic Observations Across Blocks at Site RG086IN2

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	P0760 Maize	XL5939 Maize	XL6158 Maize	P1105 Maize
V2-V5 Growth Stages (Evaluated 6/14/2018)							
Insect Damage	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	FAW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	CS	None	None	None	None	None	None
	ES	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	MPI	None	None	None	None	None	None
	WD	Slight	Slight	Slight	Slight	Slight	Slight
V7-V9 Growth Stages (Evaluated 7/2/2018)							
Insect Damage	AM	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	FB	None	None	None	None	None	None
Pathogen Stressor	AN	Slight	Slight	Slight	Slight	Slight	Slight
	ES	Slight	Slight	Slight	Slight	Slight	Slight
	SW	None	None	None	None	None	None-Slight
Abiotic Stressor	HS	None	None	None	None	None	None
	SCP	Slight-Moderate	None-Slight	None-Slight	None-Moderate	None-Moderate	None-Moderate
	SS	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/3/2018)							
Insect Damage	FAW	None	None-Slight	None	None	None-Slight	None-Slight
	JP	None	None	None	None	None-Slight	None
	RW	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None-Slight	None	None	None	None-Slight	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	SCP	None	None	None	None	None-Slight	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 8/31/2018)							
Insect Damage	AP	None	None	None	None	None	None
	CEW	Slight	Slight	Slight	Slight	Slight	Slight
	RW	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	Slight	Slight	None-Slight	None-Slight	None-Slight	None-Slight
	RSC	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), fall armyworms (FAW), flea beetles (FB), Japanese beetles (JP), and rootworm (RW). Pathogen stressors consisted of anthracnose (AN), corn stunt (CS), eyespot (ES), grey leaf spot (GLS), northern leaf blight (NLB), common maize rust (RSC), and Stewart's wilt (SW). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), soil compaction (SCP), sun scald (SS), wind damage (WD), waterlogging (WL), and maintenance and non-target pesticide injury (MPI).

Table 157. Biotic and Abiotic Observations Across Blocks at Site RG086MN1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	P0604 Maize	2R602 Maize	35A52 Maize	P0760 Maize
V2-V5 Growth Stages (Evaluated 6/5/2018)							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
Pathogen Stressor	CS	None	None	None	None	None	None
	ES	None	None	None	None	None	None
	GW	None	None	None	None	None	None
Abiotic Stressor	CD	None	None	None	None	None	None
	FR	None	None	None	None	None	None
	HL	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/25/2018)							
Insect Damage	AM	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	FB	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HL	Slight	Slight	Slight	Slight	Slight	Slight
	MPI	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/1/2018)							
Insect Damage	CEW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None-Slight	None	None-Slight
	NLB	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	HL	Slight	Slight	Slight	Slight	Slight	Slight
	ND	None-Slight	None-Slight	None	None-Slight	None	None-Slight
	WL	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 9/8/2018)							
Insect Damage	AP	None	None	None	None	None	None
	CEW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
Pathogen Stressor	AN	None-Slight	None-Slight	None-Slight	Slight	Slight	Slight
	GLS	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	HL	Slight	Slight	Slight	Slight	Slight	Slight
	ND	None-Slight	None-Slight	None	None-Slight	None	None-Slight
	WL	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), flea beetles (FB), and rootworm (RW). Pathogen stressors consisted of anthracnose (AN), corn stunt (CS), eyespot (ES), grey leaf spot (GLS), Goss' bacterial wilt (GW), northern leaf blight (NLB), and common maize rust (RSC). Abiotic stressors consisted of cold stress (CD), drought (DR), frost (FR), hail (HL), nutrient deficiency (ND), waterlogging (WL), and maintenance and non-target pesticide injury (MPI).

Table 158. Biotic and Abiotic Observations Across Blocks at Site RG086NE1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	2R602 Maize	P0993 Maize	BK6076 Maize	P1105 Maize
V2-V5 Growth Stages (Evaluated 6/7/2018)							
Insect Damage	FB	None	None	None	None	None	None
	JB	None	None	None	None	None	None
	SB	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	ES	None	None	None	None	None	None
	GW	None	None	None	None	None	None
Abiotic Stressor	MPI	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	SCR	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 7/24/2018)							
Insect Damage	GH	None	None	None	None	None	None
	SM	None	None	None	None	None	None
	WBC	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	GW	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	HL	Slight	Slight	Slight	Slight	Slight	Slight
	WD	Slight	Slight	Slight	Slight-Moderate	Slight	Slight-Moderate
	WL	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 9/27/2018)							
Insect Damage	CEW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None-Slight
	SM	None	None	None	None	None	None
Pathogen Stressor	BSR	None	None	None	None	None	None
	ER	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	HL	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None-Moderate	None-Moderate	None-Slight	None-Moderate	None-Slight	Moderate

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of corn ear worm (CEW), European corn borer (ECB), flea beetles (FB), grasshoppers (GH), Japanese beetles (JB), stink bugs (SB), spider mites (SM), and western bean cutworms (WBC). Pathogen stressors consisted of anthracnose (AN), bacterial stalk rot of maize (BSR), ear rot (ER), eyespot (ES), Goss' bacterial wilt (GW), and common maize rust (RSC). Abiotic stressors consisted of hail (HL), nutrient deficiency (ND), soil crusting (SCR), wind damage (WD), waterlogging (WL), and maintenance and non-target pesticide injury (MPI). V7-V9 biotic and abiotic observations were not collected due to a collection error.

Table 159. Biotic and Abiotic Observations Across Blocks at Site RG086ON3B

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	P0604 Maize	2R602 Maize	P0760 Maize	BK5883 Maize
V2-V5 Growth Stages (Evaluated 6/13/2018)							
Insect Damage	AM	None	None	None	None	None	None
	BB	None-Slight	None-Slight	None-Slight	None-Slight	None	None
	SG	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	ND	None-Slight	None-Slight	None-Slight	None-Slight	None	None-Moderate
	PM	None-Slight	None	None-Slight	None	None-Slight	None
	WD	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/30/2018)							
Insect Damage	AM	None	None	None	None	None	None
	BB	None	None	None	None	None	None
	WBC	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	MPI	None	None-Slight	None	None	None	None
	ND	None	None-Slight	None	None	None-Slight	None
	SCP	None-Slight	None-Slight	None	None-Moderate	None-Slight	None
R1-R2 Growth Stages (Evaluated 8/2/2018)							
Insect Damage	AM	None	None	None	None	None-Slight	None
	AP	None	None	None	None	None	None
	JP	None-Slight	Slight	Slight	None-Slight	None-Slight	Slight
Pathogen Stressor	GLS	Slight	Slight	Slight	None-Slight	None-Slight	None-Slight
	NLB	None	None-Slight	None	None	None-Slight	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	DR	None-Slight	None-Slight	Slight	None-Slight	None-Slight	Slight
	HS	None	None	None	None	None	None
	ND	Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
R3-R6 Growth Stages (Evaluated 9/27/2018)							
Insect Damage	AP	Slight-Moderate	Slight	Slight-Moderate	None-Moderate	Slight	Moderate
	ECB	None	None-Slight	None-Slight	None-Slight	None-Slight	None
	FAW	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight-Moderate	Slight-Moderate	Slight
	NLB	Slight-Moderate	Slight-Moderate	Slight	Slight	Slight	Slight
	SMT	None-Slight	None-Slight	None-Slight	None	None	None-Slight
Abiotic Stressor	AN	None	None	None	None	None	None
	HL	None	None	None	None	None	None
	WD	Slight-Severe	None-Severe	None-Slight	Slight-Moderate	None-Moderate	None-Slight

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), billbugs (BB), European corn borer (ECB), fall armyworms (FAW), Japanese beetles (JP), slugs (SG), and western bean cutworms (WBC). Pathogen stressors consisted of eyespot (ES), grey leaf spot (GLS), northern leaf blight (NLB), common maize rust (RSC), smut (SMT), and Stewart's wilt (SW). Abiotic stressors consisted of animal damage (AN), drought (DR), hail (HL), heat stress (HS), nutrient deficiency (ND), mechanical damage/purple leaf tips (PM), soil compaction (SCP), wind damage (WD), and maintenance and non-target pesticide injury (MPI). An R3-R6 smut rating was not available from one plot of 2R602 due to a collection error.

Table 160. Biotic and Abiotic Observations Across Blocks at Site RG086PA1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	P0604 Maize	2R602 Maize	35A52 Maize	BK5883 Maize
V2-V5 Growth Stages (Evaluated 6/18/2018)							
Insect Damage	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	FB	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	SCR	None	None	None	None	None	None
	SS	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 7/9/2018)							
Insect Damage	ECB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
	JP	None	None-Slight	None-Slight	None-Slight	None-Slight	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	DR	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
	HS	None	None	None	None	None	None
	SS	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/6/2018)							
Insect Damage	FAW	None-Moderate	None	None	None	None	None
	JP	None-Slight	None	None-Slight	None-Slight	None-Slight	None-Slight
	RW	None	None	None	None	None-Slight	None-Slight
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	None-Slight
	NLB	None	None	None	None-Slight	None	None
	RSC	None	None-Slight	None-Slight	None	None-Slight	None
Abiotic Stressor	HS	None	None	None	None	None	None
	SS	None	None	None	None	None	None
	WL	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 9/7/2018)							
Insect Damage	CEW	None	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
	ECB	None	None	None	None	None	None
	GH	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	Slight
Pathogen Stressor	AN	Slight-Moderate	Moderate	Moderate	Slight-Moderate	Slight-Moderate	Slight
	GLS	Slight-Moderate	Moderate	Slight	Slight	Slight-Moderate	Slight
	RSC	Slight-Moderate	Slight-Moderate	Slight	Slight	Slight	Slight
Abiotic Stressor	HS	None	None	None	None	None	None
	SS	None	None	None	None	None	None
	WD	None	None	None-Slight	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), fall armyworms (FAW), flea beetles (FB), grasshoppers (GH), Japanese beetles (JP), and rootworm (RW). Pathogen stressors consisted of anthracnose (AN), grey leaf spot (GLS), northern leaf blight (NLB), common maize rust (RSC), and Stewart's wilt (SW). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), soil crusting (SCR), sun scald (SS), wind damage (WD), and waterlogging (WL).

Table 161. Biotic and Abiotic Observations Across Blocks at Site RG086TX1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	BK5883 Maize	P0928 Maize	P1151 Maize	P1197 Maize
V2-V5 Growth Stages (Evaluated 5/30/2018)							
Insect Damage	AM	None	None	None	None	None	None
	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None-Slight	None	None-Slight	None-Slight	None-Slight	None-Slight
	WD	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/20/2018)							
Insect Damage	AM	Slight	Slight	Moderate	Slight	Slight-Moderate	Moderate
	AP	None	None	None	None	None	None
	CEW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None-Slight	None-Slight	Slight	None-Slight	None-Slight	None-Slight
	WD	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 7/12/2018)							
Insect Damage	AM	Slight	Slight	Slight-Moderate	Slight	Slight-Moderate	Slight-Moderate
	AP	None	None	None	None	None	None
	CEW	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
	SMT	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None-Slight	None	Slight	None	None-Slight	None-Slight
	WD	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 10/2/2018)							
Insect Damage	AP	None	None	None	None	None	None
	CEW	None	None	None	None	None	None
	SM	None	None	None	None	None	None
Pathogen Stressor	ER	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
	SMT	None-Slight	None-Slight	None-Slight	None-Slight	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), black cutworms (BCW), corn ear worm (CEW), and spider mites (SM). Pathogen stressors consisted of anthracnose (AN), ear rot (ER), eyespot (ES), grey leaf spot (GLS), common maize rust (RSC), and smut (SMT). Abiotic stressors consisted of drought (DR), heat stress (HS), and wind damage (WD).

Table 162. Biotic and Abiotic Observations Across Blocks at Site RG086TX7

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP23211 Maize	Control Maize	35A52 Maize	P1105 Maize	P1151 Maize	P1197 Maize
V2-V5 Growth Stages (Evaluated 6/6/2018)							
Insect Damage	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	FAW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	MDMV	None	None	None	None	None	None
	WSMV	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None-Slight	None	None	None
	HS	None	None	None	None	None	None
	SCR	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/29/2018)							
Insect Damage	AP	None	None	None	None	None	None
	FAW	Slight	Slight	Slight	Slight	Slight	Slight
	SM	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	MDMV	None	None	None	None	None	None
	SLB	None	None	None	None	None	None
Abiotic Stressor	HS	None	None	None	None	None	None
	MPI	None	None	None	None	None	None
	ND	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 7/18/2018)							
Insect Damage	AM	Slight	Slight	Slight	Slight	Slight	Slight
	CEW	Slight	Slight	Slight	Slight	Slight	Slight
	SM	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	GW	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	HS	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 8/21/2018)							
Insect Damage	CEW	Slight	Slight	Slight	Slight	Slight	Slight
	SCB	None	None	None	None	None	None
	SM	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GW	None	None	None	None	None	None
	WSMV	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), black cutworms (BCW), corn ear worm (CEW), fall armyworms (FAW), southwestern corn borer (SCB), and spider mites (SM). Pathogen stressors consisted of anthracnose (AN), grey leaf spot (GLS), Goss' wilt (GW), maize dwarf mosaic virus (MDMV), common maize rust (RSC), southern leaf blight of maize (SLB), and wheat streak mosaic virus (WSMV). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), soil crusting (SCR), wind damage (WD), and maintenance and non-target pesticide injury (MPI). V7-V9, R1-R2, and R3-R6 data were not available from one block due to herbicide drift.

Experiment B – 2015-2017 Field Insect and Disease Observations

DP23211 Maize has been field tested in the United States and Puerto Rico over 5 years, as authorized by USDA-APHIS permits and notifications (Appendix 1. DP23211 Maize USDA Release Permits, Notifications, and Planted Acreage). For each trial, a survey of the naturally occurring insects and diseases and any unexpected differences in the response of DP23211 maize as compared to the control line (near-isoline, non-GE maize lines) were recorded by experienced plant breeders and field staff at least every four weeks. The plant breeders and field staff were familiar with plant pathology and entomology and recorded the severity of any insect or disease in the field. These observations provide a means to determine if DP23211 maize will respond differently from conventional maize lines to insects or diseases in the environment.

A summary of the naturally-occurring insects noted in field observations and any unexpected differences seen between DP23211 maize and control lines is presented in Table 161. A summary of diseases present in the field observations is presented in Table 162.

The following scale was used to evaluate disease or insect injury in DP23211 maize and control lines (Table 162 and Table 161).

Range of Severity in DP23211 Maize:

- Mild – very little disease or insect injury (<10%) visible
- Moderate – noticeable plant tissue damage (10% - 30%)
- Severe – significant plant tissue damage (>30%)

Abiotic stressor field observations were recorded at all United States and Puerto Rico locations and are presented in Table 163.

In every case, DP23211 maize did not exhibit any unexpected responses to naturally-occurring insects, diseases or abiotic stressors as compared to the control line.

Table 163. Observations of Insects Present and Comparison Between DP23211 Maize and Control

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2015	15-096-101n	PR	Salinas	Aphids (Aphididae)	Mild	None
				Other - Chrysoperla	Mild	None
				Other	Moderate	None
	15-309-101n	HI	Kauai	Grasshoppers (Orthoptera)	Mild	None
				Mites (Acari)	Mild	None
2016	16-039-106n	HI	Kauai	Leafhopper (Cicadellidae)	Mild	None
				Mites (Acari)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
	16-293-101rm-a1	HI	Kauai	Chinese rose beetle (Adoretus sinicus)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Leafhopper (Cicadellidae)	Mild	None
				Mites (Acari)	Mild	None
				Other - Moth	Mild	None
		PR	Salinas	Grasshoppers (Orthoptera)	Mild	None
				Leafhopper (Cicadellidae)	Mild - Moderate	None
				Other - Spidermite	Mild	None
				Aphids (Aphididae)	Mild - Moderate	None
				Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Cornsilk fly (Euxesta stigmatias)	Mild	None
				Corn earworm (Helicoverpa zea)	Moderate	None
				Corn sap beetle (Carpophilus spp.)	Moderate	None

Table 161. Observations of Insects Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-038-105rm-a1	IL	Bureau	Aphids (Aphididae)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
			Champaign	Corn flea beetle (Chaetochema pulicaria)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
			McDonough	Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild - Moderate	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
		IA	Polk	Japanese beetle (Popillia japonica)	Mild	None
		NE	York	Aphids (Aphididae)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Mites (Acari)	Mild - Moderate	None
				Western bean cutworm (Striacosta albicosta)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
		TN	Obion	Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Corn earworm (Helicoverpa zea)	Mild - Moderate	None
				Spotted cucumber beetle (Diabrotica undecimpunctata)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
	17-264-103rm	HI	Kauai	Chinese rose beetle (Adoretus sinicus)	Mild	None
	17-311-102rm	PR	Salinas	Aphids (Aphididae)	Mild	None
				Armyworms (Spodoptera spp.)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Cornsilk fly (Euxesta stigmatias)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Leafhopper (Cicadellidae)	Mild	None
				Other - Spidermite	Mild	None

Table 161. Observations of Insects Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-264-103rm	HI	Kauai	Chinese rose beetle (Adoretus sinicus)	Mild	None
		IL	Shelby	Armyworms (Spodoptera spp.)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
		IA	Greene	European corn borer (Ostrinia nubilalis)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
		KS	Pawnee	Corn earworm (Helicoverpa zea)	Mild - Moderate	None
				European corn borer (Ostrinia nubilalis)	Mild	None
				Other - Southwestern cornborer	Mild	None
		MO	Butler	Armyworms (Spodoptera spp.)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
		NE	York	Aphids (Aphididae)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Western bean cutworm (Striacosta albicosta)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
		NJ	Hunterdon	Black cutworm (Agrotis ipsilon)	Mild	None
		PA	Lehigh	Corn earworm (Helicoverpa zea)	Mild	None
				European corn borer (Ostrinia nubilalis)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
		TX	Tom Green	Armyworms (Spodoptera spp.)	Mild - Moderate	None

Table 161. Observations of Insects Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-033-102rm	IL	Bureau	Aphids (Aphididae)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
			Champaign	Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
			Coles	Armyworms (Spodoptera spp.)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
			Piatt	Corn earworm (Helicoverpa zea)	Mild	None
				Grasshoppers (Orthoptera)	Mild - Moderate	None
				Japanese beetle (Popillia japonica)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild - Moderate	None
		IN	Benton	Northern corn rootworm (Diabrotica barberi)	Moderate	None
			Tipton	Northern corn rootworm (Diabrotica barberi)	Mild	None
		IA	Bremer	Corn earworm (Helicoverpa zea)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
				Japanese beetle (Popillia japonica)	Moderate	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Moderate	None
			Linn	Japanese beetle (Popillia japonica)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
				Other - Southern Corn Rootworm	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild - Moderate	None
			Polk	Japanese beetle (Popillia japonica)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None

Table 161. Observations of Insects Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-033-102rm	MN	Blue Earth	Western corn rootworm (Diabrotica virgifera virgifera)	Mild - Moderate	None
			Swift	Western corn rootworm (Diabrotica virgifera virgifera)	Moderate	None
		NE	Platte	Western corn rootworm (Diabrotica virgifera virgifera)	Moderate	None
			York	Aphids (Aphididae)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
				European corn borer (Ostrinia nubilalis)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Spotted cucumber beetle (Diabrotica undecimpunctata)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
				Western bean cutworm (Striacosta albicosta)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild - Moderate	None
		SD	Brookings	Northern corn rootworm (Diabrotica barberi)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild - Severe	None
		TN	Obion	Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Corn earworm (Helicoverpa zea)	Mild - Moderate	None
				European corn borer (Ostrinia nubilalis)	Mild	None
				Other - Southwestern Corn Borer (Diatraea grandiosella)	Mild - Moderate	None
				Spotted cucumber beetle (Diabrotica undecimpunctata)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
		TX	Hale	Corn earworm (Helicoverpa zea)	Moderate	None
				Two-spotted spider mite (Tetranychus urticae)	Mild	None
		WI	Rock	Northern corn rootworm (Diabrotica barberi)	Mild	None
	18-033-103rm	IL	Bureau	Aphids (Aphididae)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
			Champaign	Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
			Coles	Armyworms (Spodoptera spp.)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
		IN	Tipton	Northern corn rootworm (Diabrotica barberi)	Mild	None

Table 161. Observations of Insects Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-033-103rm	IA	Linn	Japanese beetle (Popillia japonica)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
				Other - Southern Corn Rootworm	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild - Moderate	None
			Polk	Japanese beetle (Popillia japonica)	Mild	None
		KS	Hodgeman	Corn earworm (Helicoverpa zea)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Mites (Acari)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
		MO	Scott	Aphids (Aphididae)	Mild	None
				Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Corn earworm (Helicoverpa zea)	Mild - Moderate	None
				Grasshoppers (Orthoptera)	Mild	None
				Other - Southwestern Corn Borer (Diatraea grandiosella)	Mild - Moderate	None
				Spotted cucumber beetle (Diabrotica undecimpunctata)	Mild	None
				Thrips (Frankliniella spp.)	Mild	None
		NE	Lancaster	Aphids (Aphididae)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
			York	Corn earworm (Helicoverpa zea)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
				Western bean cutworm (Striacosta albicosta)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild - Moderate	None
		TN	Obion	Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Corn earworm (Helicoverpa zea)	Mild - Moderate	None
				European corn borer (Ostrinia nubilalis)	Mild	None
				Other - Southwestern Corn Borer (Diatraea grandiosella)	Mild - Moderate	None
				Spotted cucumber beetle (Diabrotica undecimpunctata)	Mild	None
				Stink bugs (Pentatomidae)	Mild	None
		TX	Hale	Corn earworm (Helicoverpa zea)	Moderate	None
				Two-spotted spider mite (Tetranychus urticae)	Mild	None

Table 161. Observations of Insects Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-102-102rm	IN	Clinton	Armyworms (Spodoptera spp.)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
		SD	Brookings	Aphids (Aphididae)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
	18-192-107rm	PR	Salinas	Aphids (Aphididae)	Mild	None
				Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Chinch Bugs (Lygaeidae)	Moderate	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Cornsilk fly (Euxesta stigmatias)	Mild	None
				Leafhopper (Cicadellidae)	Mild - Moderate	None
				Other - Chrysoperla	Moderate	None
				Other - Spidermite	Mild	None
	18-192-110rm	PR	Salinas	Aphids (Aphididae)	Mild	None
				Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Chinch Bugs (Lygaeidae)	Moderate	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Cornsilk fly (Euxesta stigmatias)	Mild	None
				Leafhopper (Cicadellidae)	Mild - Moderate	None
				Other - Chrysoperla	Moderate	None
				Other - Spidermite	Mild	None
	18-192-110rm	PR	Salinas	Aphids (Aphididae)	Mild	None
				Armyworms (Spodoptera spp.)	Mild - Moderate	None
				Chinch Bugs (Lygaeidae)	Moderate	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Cornsilk fly (Euxesta stigmatias)	Mild	None
				Leafhopper (Cicadellidae)	Mild - Moderate	None
				Other - Chrysoperla	Moderate	None
				Other - Spidermite	Mild	None

Table 164. Observations of Diseases Present and Comparison Between DP23211 Maize and Control Maize

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2015	15-096-101n	PR	Salinas	Bacterial leaf blight and stalk rot (Pseudomonas avenae subsp. avenae)	Moderate	None
				Other - Fungus	Mild	None
2016	16-039-106n	HI	Kauai	Maize stripe virus (Tenuivirus)	Mild	None
	16-293-101rm-a1	HI	Kauai	Maize stripe virus (Tenuivirus)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
		PR	Salinas	Other - Bacterial	Mild	None
				Other - Fungus	Mild	None
				Other - Virus	Mild	None
				Southern corn leaf blight (Bipolaris maydis)	Mild	None
2017	17-038-105rm-a1	IL	Bureau	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
			Champaign	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
			McDonough	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Gray spot (Pyricularia spp.)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
				Northern corn leaf spot (Bipolaris zeicola)	Mild	None
		IN	Tipton	Southern corn leaf blight (Bipolaris maydis)	Mild	None
		IA	Dallas	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
			Polk	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		NE	York	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Southern rust (Puccinia polysora)	Mild	None
		TN	Obion	Brown spot (Physoderma maydis)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
				Southern rust (Puccinia polysora)	Mild - Moderate	None
				Gray leaf spot (Cercospora zeae-maydis)	Moderate	None

Table 162. Observations of Diseases Present and Comparison Between DP23211 Maize and Control Maize (continued)

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-264-103rm	HI	Kauai	Common rust (Puccinia sorghi)	Mild	None
	17-311-102rm	PR	Salinas	Other - Bacteria	Mild	None
				Other - Fungus	Mild	None
				Other - Virus	Mild	None
				Southern corn leaf blight (Bipolaris maydis)	Mild	None
	17-264-103rm	HI	Kauai	Common rust (Puccinia sorghi)	Mild	None
2018	18-016-102rm		Shelby	Gray leaf spot (Cercospora zeae-maydis)	Moderate	None
		IA	Greene	Anthracnose stalk rot (Colletotrichum graminicola)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Fusarium ear rot (Fusarium subglutinans)	Mild	None
				Fusarium stalk rot (Fusarium avenaceum or Fusarium subglutinans)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
			Jefferson	Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		KS	Pawnee	Common rust (Puccinia sorghi)	Mild - Moderate	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		MN	Freeborn	Eye spot of corn (Aureobasidium zeae)	Mild	None
		MO	Butler	Anthracnose stalk rot (Colletotrichum graminicola)	Mild	None
				Gibberella ear (Gibberella zeae)	Mild	None

Table 162. Observations of Diseases Present and Comparison Between DP23211 Maize and Control Maize (continued)

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-016-102rm	NE	York	Anthracnose stalk rot (Colletotrichum graminicola)	Moderate	None
				Aspergillus ear rot (Aspergillus spp.)	Moderate	None
				Common smut (Ustilago maydis)	Moderate	None
				Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Gibberella ear (Gibberella zeae)	Moderate	None
				Gibberella stalk rot (Gibberella zeae)	Moderate	None
				Fusarium ear rot (Fusarium subglutinans)	Moderate - Severe	None
				Fusarium stalk rot (Fusarium avenaceum or Fusarium subglutinans)	Moderate	None
		NJ	Hunterdon	Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
		PA	Lehigh	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild - Moderate	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
				Anthracnose stalk rot (Colletotrichum graminicola)	Moderate - Severe	None
		TX	Tom Green	Common smut (Ustilago maydis)	Mild	None
		WI	Walworth	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
				Other - tar spot	Mild	None
	18-033-102rm	IL	Bureau	Brown spot (Physoderma maydis)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild - Moderate	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
				Other - Tar Spot (Phyllachora maydis)	Mild - Moderate	None
			Champaign	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild - Moderate	None

Table 162. Observations of Diseases Present and Comparison Between DP23211 Maize and Control Maize (continued)

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-033-102rm	IL	Coles	Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
			Piatt	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Gray spot (Pyricularia spp.)	Moderate	None
		IN	Benton	Northern corn leaf blight (Exserohilum turcicum)	Mild - Moderate	None
			Tipton	Northern corn leaf blight (Exserohilum turcicum)	Mild - Moderate	None
				Northern corn leaf spot (Bipolaris zeicola)	Mild	None
		IA	Bremer	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
			Jasper	Gray leaf spot (Cercospora zeae-maydis)	Mild	None
			Linn	Anthracnose stalk rot (Colletotrichum graminicola)	Mild	None
				Brown spot (Physoderma maydis)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
			Polk	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
		NE	York	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gosss Wilt (Clavibacter michiganensis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		SD	Brookings	Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		TN	Obion	Brown spot (Physoderma maydis)	Moderate	None
				Gray leaf spot (Cercospora zeae-maydis)	Moderate	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
				Southern rust (Puccinia polysora)	Mild	None
		TX	Hale	Fusarium ear rot (Fusarium subglutinans)	Mild	None
		WI	Rock	Common smut (Ustilago maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None

Table 162. Observations of Diseases Present and Comparison Between DP23211 Maize and Control Maize (continued)

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-033-103rm	IL	Bureau	Brown spot (Physoderma maydis)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild - Moderate	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
				Other - Tar Spot (Phyllachora maydis)	Mild - Moderate	None
			Champaign	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild - Moderate	None
			Coles	Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		IN	Tipton	Northern corn leaf blight (Exserohilum turcicum)	Mild - Moderate	None
				Northern corn leaf spot (Bipolaris zeicola)	Mild	None
		IA	Jasper	Gray leaf spot (Cercospora zeae-maydis)	Mild	None
			Linn	Anthracnose stalk rot (Colletotrichum graminicola)	Mild	None
				Brown spot (Physoderma maydis)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
			Madison	Common rust (Puccinia sorghi)	Mild	None
			Polk	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
		MO	Scott	Brown spot (Physoderma maydis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild - Moderate	None
				Northern corn leaf spot (Bipolaris zeicola)	Mild	None
				Southern rust (Puccinia polysora)	Moderate	None
		NE	Lancaster	Bacterial leaf blight and stalk rot (Pseudomonas avenae subsp. avenae)	Mild	None
				Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gibberella stalk rot (Gibberella zeae)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
			York	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Gosss Wilt (Clavibacter michiganensis)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None

Table 162. Observations of Diseases Present and Comparison Between DP23211 Maize and Control Maize (continued)

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-033-103rm	TN	Obion	Brown spot (Physoderma maydis)	Moderate	None
				Gray leaf spot (Cercospora zeae-maydis)	Moderate	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
		TX	Hale	Fusarium ear rot (Fusarium subglutinans)	Mild	None
	18-102-102rm	IN	Clinton	Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Gray spot (Pyricularia spp.)	Mild	None
		IA	Howard	Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		SD	Brookings	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
	18-192-107rm	PR	Salinas	Common rust (Puccinia sorghi)	Mild	None
				Other - Bacteria	Mild	None
				Other - Fungus	Mild	None
				Other - Virus	Mild	None
				Southern corn leaf blight (Bipolaris maydis)	Mild	None
	18-192-110rm	PR	Salinas	Common rust (Puccinia sorghi)	Mild	None
				Other - Bacteria	Mild	None
				Other - Fungus	Mild	None
				Other - Virus	Mild	None
				Southern corn leaf blight (Bipolaris maydis)	Mild	None

Table 165. Observations of Abiotic Stressors Present and Comparison Between DP23211 Maize and Control Maize

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-038-105rm-a1	IL	Bureau	Some plants were leaning due to high winds from a storm the prior night	Mild	None
			Champaign	wet before emergence then turning hot and dry	Moderate	None
				drought due to no significant rain in June	Severe	None
				drought early with a storm with high winds pre-flowering	Moderate	None
				drought early and continued	Moderate	None
				drought , wind before flowering	Mild	None
		IN	Tipton	Heavy rain and wind	Moderate	None
				High heat and dry condition.	Mild	None
		IA	Polk	Hail damage	Mild	None
				Wind damage	Mild	None
		NE	York	Corn endured some high heat temperatures in mid July.	Mild	None
				t-storms with high winds occurred early in the morning of Aug. 20 and susceptible genetics have some lod	Mild	None
	17-264-103rm	HI	Kauai	Nutrient deficiency	Mild	None
2018	17-264-103rm	HI	Kauai	Nutrient deficiency	Mild	None
	18-016-102rm	IL	Clinton	high temperatures	Mild	None
				dry weather	Mild	None
				extreme temperatures	Severe	None
			Shelby	drought	Mild	None
				high wind	Mild	None
		IA	Greene	High winds likely contributed to the severity of lodging in the trial	Mild	None
		MO	Butler	High winds, saturated soils, and hail caused torn leaves and purple stems.	Moderate	None
				Drought stress and heat stress were present	Mild	None
		NE	York	high wind	Mild	None
				hail and wind damage	Mild - Severe	None
				wind and hail in august	Severe	None
		NJ	Hunterdon	Wet Soil Conditions	Mild	None
		PA	Lehigh	Higher than average moisture with poor drying conditions	Severe	None
		TX	Tom Green	Extreme heat	Mild	None

Table 163. Observations of Abiotic Stressors Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-033-102rm	IL	Bureau	A high wind event occurred during late June that caused plants to lodge in the field.	Moderate	None
				This location experienced a wind event prior to flowering.	Moderate	None
			Coles	Wind damage	Mild	None
		IA	Jasper	Wind damage	Mild	None
			Linn	Excess Moisture	Mild	None
			Polk	Wind damage	Mild	None
				Hail damage	Mild	None
		MN	Blue Earth	The month of June had above normal rainfall.	Mild	None
			Swift	Some unevenness due to excessive water during June.	Mild	None
		NE	York	Locations sustained hail damage from Aug. 6 evening storm. Upper canopy has moderate leaf shredding.	Moderate	None
		SD	Brookings	root lodging	Moderate	None
		TN	Obion	High winds	Mild	None
				Extreme hot temps	Moderate	None
		TX	Hale	Managed Drought stress	Moderate	None
		WI	Rock	Wind event late June caused some basal snap.	Mild	None
	18-033-103rm	CA	Yolo	Wind damage	Mild	None
		IL	Bureau	A wind event came through the area a few weeks ago and lodged certain parts of the field.	Moderate	None
				A high wind event occurred during late June that caused plants to lodge in the field.	Moderate	None
				This location experienced a wind event prior to flowering.	Moderate	None
			Coles	Wind damage	Mild	None
		IA	Jasper	Wind damage	Mild	None
			Linn	Excess Moisture	Mild	None
			Madison	Wind damage	Mild	None
				Wind and hail damage	Severe	None
			Polk	Wind damage	Mild	None
				Hail damage	Mild	None
		KS	Hodgeman	Drought stress. Severe. This is expected. Nitrogen and Potassium Deficiency, Very Mild. Root lodging from heavy wind event.	Moderate	None
		NE	Lancaster	There is some drought stress as location has only received about 1.0" of rain since July 1. Crop is maturating at a faster pace.	Mild	None
				Crop endured some drought stress during late June through July.	Moderate	None
			York	Locations have sustained hail damage from Aug 6 evening storms.	Moderate	None
				Field has sustained hail damage from early Aug storms.	Moderate	None
		TN	Obion	High winds	Mild	None
				Extreme hot temps	Moderate	None
		TX	Hale	Managed Drought stress	Moderate	None

Table 163. Observations of Abiotic Stressors Present and Comparison Between DP23211 Maize and Control (continued)

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2018	18-102-102rm	IN	Clinton	Wind and rain	Mild	None
		IA	Howard	We have had 8-10 inches of rain in June.	Mild	None
		SD	Brookings	Early freeze	Severe	None
				Hard freeze	Severe	None
	18-192-107rm	HI	Kauai	High winds	Mild	None
	18-192-110rm	HI	Kauai	High winds	Mild	None
2019	19-030-101rm	NE	York	Emergence has been slow due to cool and wet spring.	Mild	None

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PIONEER®

Petition for the Determination of Nonregulated Status for Insect Resistant and Herbicide-Tolerant DP23211 Maize

Volume 2 - Appendices

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OECD Unique Identifier: DP-Ø23211-2

Submission date: July 20, 2020

Does Not Contain Confidential Business Information

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article, DP-Ø23211-2 maize (DP23211) not be regulated under 7 CFR §340.

Sally A. Catron 7/20/2020

Sally A. Catron Date

Release of Information

Pioneer Hi-Bred International, Inc. (“Pioneer”, a member of the Corteva Agriscience Group of Companies), is submitting the information in this assessment for review by the USDA as part of the regulatory process. By submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C. section 522 or USDA’s implementation regulations (7 CFR §340). In the event that USDA receives a FOIA request covering all or some of the information in this submission, Pioneer expects that, in advance of the release of the document(s), USDA will provide Pioneer with a copy of the material proposed to be released and the opportunity to object to the release of any information based upon appropriate legal grounds, e.g., responsiveness, confidentiality, or otherwise. Pioneer expects that no information that has been identified as confidential business information (CBI) will be provided to any third party. Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without prior notice and consent from Pioneer.

Certification

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article, DP-Ø23211-2 maize (DP23211) not be regulated under 7 CFR §340.

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners, which are favorable and unfavorable to the petition.

Sally A. Catron 7/20/2020

Sally A. Catron Date
United States Seeds Regulatory Affairs Leader
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Appendix A. Bioinformatics Analysis of DvSSJ1 Specificity

Overview

A bioinformatics approach was used to assess how conserved the smooth septate junction protein 1 (*ssj1*) gene sequence is across different organisms with varied evolutionary distance from western corn rootworm (WCR, *Diabrotica virgifera virgifera*).

The sequences of the *ssj1* homologs from twenty species, representing four families within the order Coleoptera, four families within the order Lepidoptera, and two additional non-target organisms were compared to a 210-base pair (210-bp) sequence from the *dvssj1* gene (referred to as 210-bp *dvssj1* sequence) to determine the percent similarity, number of single nucleotide polymorphisms (SNPs), and the number of 21 nucleotide (21-nt) matches.

The closest sequence match (percent identity to the 210-bp *dvssj1* sequence) was *ssj1* from WCR (*WCR)*, which as intended had a 100% sequence match, 0 SNPs, and 190 21-nt matches to the 210-bp *dvssj1* sequence. The *ssj1* homologous gene from the closely related species, northern corn rootworm (NCR; *D. barberi*), shared 97.1% identity with the 210-bp *dvssj1* sequence, with 6 SNPs and 135 21-nt matches. The *ssj1* homologous gene from the southern corn rootworm (SCR; *SCR*) shared 92.9% identity with the 210-bp *dvssj1* sequence, with 15 SNPs and 79 21-nt matches. The *ssj1* homologous genes from all of the non-*Diabrotica* species analyzed had lower percent identity with the 210-bp *dvssj1* sequence, an increased number of SNPs, and zero 21-nt matches.

Materials and Methods

The sequences of the *ssj1* homologs from 13 organisms with varied evolutionary distance from WCR were obtained from published data (Hu *et al.*, 2016)(Table 1).

The transcriptomes of six additional insects, including the CDM, MBD, MWM, PLD, RVB, and SWM were assembled as previously described (Niu *et al.*, 2017) (Table 1). Briefly, complementary DNAs (cDNAs) prepared from larvae of each species were sequenced by Illumina paired-end and 454 Titanium sequencing technologies. *De novo* transcriptome assemblies were performed using the Trinity method (Grabherr *et al.*, 2011). The coding sequence of *dvssj1* gene sequence (Accession number: KU562965) was used for tblastx search transcriptome assembly. The AlignX tool of Vector NTi (Invitrogen) was used to create alignments between the 210-bp *dvssj1* sequence and the *ssj1* homologous sequences from each insect. Each alignment was inspected to determine the nucleotide percent identity over the length of the alignment. The longest contiguous match between the 210-bp *dvssj1*

sequence and each of the homologous sequences from the various insects was determined using a Perl script created by Corteva Agriscience (findExactMatches.pl) (Mirksy, 2018). All data are summarized in Table 1 and Figure 1.

Results

Using *in silico* analysis, the closest sequence match (percent identity to the 210-bp *dvssj1* sequence) was the *ssj1* homologous gene from WCR, which as intended had a 100% sequence match, 0 SNPs, and 190 21-nt matches (Table 1)The *ssj1* homologous gene from the closely related species, NCR, shared 97.1% identity with the 210-bp *dvssj1* sequence, with 6 SNPs and 135 21-nt matches. The *ssj1* homologous gene from SCR shared 92.9% identity with the 210-bp *dvssj1* sequence, with 15 SNPs and 79 21-nt matches. The *ssj1* homologous genes from the other Coleoptera within the family Chrysomelidae (CFB, SFB, and CPB) as well as species within the family Tenebrionidae, the family Coccinellidae, and the family Staphylinidae had decreasing percent identity with the 210-bp *dvssj1* sequence, ranging from 77.6 to 61.9% similarity and an increasing number of SNPs (ranging from 47 to 80). All Lepidoptera species within the four families, as well as the honey bee (*Apis mellifera)* and the insidious flower bug (*Orius insidiosus)* also had lower percent identity with the 210-bp *dvssj1* sequence, ranging from 68.1 to 60% similarity, and an increased number of SNPs (ranging from 67 to 84). There were zero of 21-nt matches observed across all of the non-*Diabrotica* species analyzed.

Conclusion

The closest genomic sequence match (percent identity to the 210-bp *dvssj1* sequence) was the *ssj1* homologous gene from WCR, which as intended had a 100% sequence match, 0 SNPs, and 190 21-nt matches. The *ssj1* homologous gene from the closely related species, NCR, shared 97.1% identity with the 210-bp *dvssj1* sequence, with 6 SNPs and 135 21-nt matches. The *ssj1* homologous gene from SCR shared 92.9% identity with the 210-bp *dvssj1* sequence, with 15 SNPs and 79 21-nt matches. The *ssj1* homologous genes from all of the non-*Diabrotica* species analyzed had lower percent identity with the 210-bp *dvssj1* sequence, an increased number of SNPs, and zero of 21-nt matches.

Table 1: List of Sequence Sources of *ssj1* Homologs

Order	Family	Species	Common Name	*ssj1* homolog Sequence Sources	Accession # or Seq. No.	Transcript Tracking No.[2]	210-bp sequences of the *ssj1* homologs Tracking No.[3]
Coleoptera	Chrysomelidae	*Diabrotica virgifera virgifera*	Western corn rootworm	(Hu *et al.*, 2016)	KU562965	Seq-1	Seq-21
	Chrysomelidae	*Diabrotica barberi*	Northern corn rootworm	US 20140275208 A1	Seq. No. 695	Seq-2	Seq-22
	Chrysomelidae	*Diabrotica undecimpunctata*	Southern corn rootworm	US 20140275208 A1	Seq. No.694	Seq-3	Seq-23
	Chrysomelidae	*Phyllotreta cruciferae*	Crucifer flea beetle	WO2016043960	Seq. No. 44	Seq-4	Seq-24
	Chrysomelidae	*Phyllotreta striolata*	Striped flea beetle	WO2016043960	Seq. No. 45	Seq-5	Seq-25
	Chrysomelidae	*Leptinotarsa decemlineata*	Colorado potato beetle	US 20140275208 A1	Seq. No. 697	Seq-6	Seq-26
	Tenebrionidae	*Tribolium castaneum*	Red flour beetle	(Hu *et al.*, 2016)	XP_008197065.1	Seq-7	Seq-27
	Tenebrionidae	*Zophobas morio*	Super worm	this study[1]	n/a	Seq-8	Seq-28
	Tenebrionidae	*Tenebrio molior*	Mealworm	this study[1]	n/a	Seq-10	Seq-30
	Staphylinidae	*Dalotia coriaria*	Rove beetle	this study[1]	n/a	Seq-11	Seq-31
	Coccinellidae	*Epilachna varivestis*	Mexican bean beetle	US 20140275208 A1	Seq. No. 696	Seq-9	Seq-29
	Coccinellidae	*Cryptolaemus montrouzieri*	Mealy bug destroyer	this study[1]	n/a	Seq-12	Seq-32

Table 1: List of Sequence Sources of *ssj1* Homologs (continued)

Order	Family	Species	Common Name	*ssj1* homolog Sequence Sources	Accession # or Seq. No.	Transcript Tracking No.[2]	210-bp sequences of the *ssj1* homologs Tracking No.[3]
Coleoptera	Coccinellidae	*Coleomegilla maculata*	Pink spotted lady beetle	US 20140275208 A1	Seq. No. 699	Seq-13	Seq-33
Lepidoptera	Nymphalidae	*Vanessa cardui*	Painted lady	this study[1]	n/a	Seq-14	Seq-34
	Crambidae	*Ostrinia nubilalis*	European corn borer	WO2016043960	Seq. No. 42	Seq-15	Seq-35
	Noctuidae	*Spodoptera frugiperda*	Fall armyworm	WO2016043960	Seq. No. 41	Seq-16	Seq-36
	Tortricidae	*Cydia pomonella*	Codling moth	this study[1]	n/a	Seq-17	Seq-37
	Noctuidae	*Helicoverpa zea*	Corn earworm	WO2016043960	Seq. No. 43	Seq-18	Seq-38
Hymentoptera	Apidae	*Apis mellifera*	Honeybee	(Hu *et al.*, 2016)	XP_003249659.1	Seq-19	Seq-39
Hemiptera	Anthocoridae	*Orius insidiosus*	Insidious flower bug	US 20140275208 A1	Seq. No. 698	Seq-20	Seq-40

[1] Figure 1 and List of sequences

[2,3] All transcripts and 210-bp sequences of the *ssj1* homologs are provided in List of Sequences

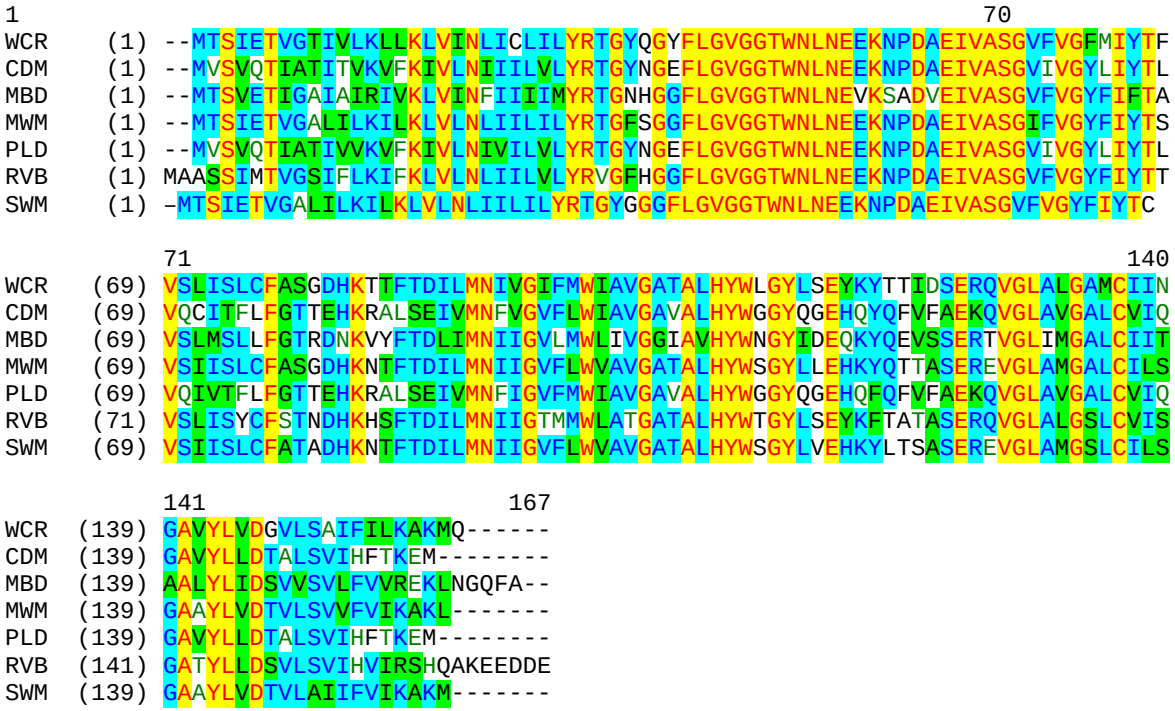

Figure 1. Amino Acid Sequence Alignment of the DvSSJ1 Protein and its Homologs from Six Species

The coding sequence of the *dvssj1* gene (KU562965) was used for tblastx search transcriptome assembly. The amino acid sequence of the DvSSJ1 protein (WCR) was used to align with the SSJ1 protein homologs from six insects. Nucleotide sequences of the six insects were used for sequence analyses (Table 1 and List of Sequences); including codling moth (CDM; *Cydia pomonella*), mealy bug destroyer (MBD; C*ryptolaemus montrouzieri*); mealworm (MWM; *Tenebrio molitor);* painted lady butterfly (PLD; *Vanessa cardui*), rove beetle (RVB; *Dalotia coriaria*); and super worm (SWM; *Zophobas morio*).

List of Sequences

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>Seq-6
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>Seq-7
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>Seq-8
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>Seq-9
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>Seq-10
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>Seq-11
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>Seq-12
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>Seq-13
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>Seq-14
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>Seq-15
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>Seq-16
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>Seq-17
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>Seq-18
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>Seq-19
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>Seq-20
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>Seq-21

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GAACGGGATACAAAGGAGAATTCCTGGGTGTCGGAGGGACATGGAACTTAAACGAAGATAAAAATCCAGACGCTGAGATGGTTGCGTCCGGAGTTTTGGTCGG

>Seq-40
GGGGACATCAAAAAATCAAAGAAAAATGGAAATGGAAACTATAATTACGATTTTTATTAAAGTCATAAAATGGGTGATAAATCTCATTGTAATCTTCTTATACCGAT
ACGGTTATTCTGGGCAATTCCTCGGTATTGGAGGAACGTGGAATTTGAATGAGGAGAAAGATGCGACAGCAGAAGTTGTCGCTTCGGGCGTCCTCGTCGGCTA

Appendix B. Comparison of the DvSSJ1 Fragment to the Human Transcriptome

Objective

A comparison of the DvSSJ1 sense and anti-sense fragment sequences (Figure 2 and Figure 3, respectively) to the sequences in the human transcriptome was performed to identify any 21-nucleotide (21-nt) exact matches, which could indicate potential off-target effects in humans.

Methods

The DvSSJ1 sense and anti-sense fragment sequences (Figure 2 and Figure 3, respectively) were compared to the human transcriptome (GRCh38, available at https://www.ncbi.nlm.nih.gov/genome/guide/human/). A Perl script written by Corteva Agriscience (find21ResidueExactMatches.pl) was used to parse the sense and anti-sense fragment sequences into all possible sequentially-overlapping 21-nt subsequences, parse each transcript in the human transcriptome into all possible sequentially-overlapping 21-nt subsequences, and then compare each DvSSJ1-derived 21-nt subsequence to each transcript-derived 21-nt subsequence. Any perfect 21-nt match between a DvSSJ1-derived subsequence and a transcript-derived subsequence would be identified by the script and written to an output file. If no perfect 21-nt matches were identified by the script, that would be written to the output file instead.

Results and Discussion

No perfect 21-nt matches were identified between the sense or anti-sense DvSSJ1 fragments and any transcript in the human transcriptome (Appendix 1 and Appendix 2, respectively). Consequently, bioinformatics analysis does not indicate the potential for off-target effects of the DvSSJ1 fragment in humans.

Figure 2. DvSSJ1 Sense Fragment Sequence

ATAATAAGTTCGATTTTTTACGAAAATGACAAGTATCGAGACTGTGGGGACCATTGTCCTGAAATTGCTGAAGTTGG
TGATCAATTTGATATGTCTCATCTTGTACCGAACCGGATATCAAGGCTACTTCTTGGGAGTAGGAGGAACCTGGAAT
CTAAACGAAGAAAAAAATCCCGATGCAGAAATTGTGGCTTCCGGCGTATTCGTAGG

Figure 3. DvSSJ1 Anti-Sense Fragment Sequence

CCTACGAATACGCCGGAAGCCACAATTTCTGCATCGGGATTTTTTTCTTCGTTTAGATTCCAGGTTCCTCCTACTCC
CAAGAAGTAGCCTTGATATCCGGTTCGGTACAAGATGAGACATATCAAATTGATCACCAACTTCAGCAATTTCAGGA
CAATGGTCCCCACAGTCTCGATACTTGTCATTTTCGTAAAAAATCGAACTTATTAT

Appendix C. Comparison of the DvSSJ1 Fragment to a Variety of Farm and Companion Animals

Objective

Comparisons of the DvSSJ1 sense and anti-sense fragment sequences (Figure 4 and Figure 5, respectively) to the sequences of a variety of farm and companion animal transcriptomes were performed to identify any 21-nucleotide (21-nt) exact matches which could indicate potential off-target effects. The animal transcriptomes evaluated in this study were pig, cattle, chicken, salmon, turkey, sheep, goat, cat, and dog.

Methods

The DvSSJ1 sense and anti-sense fragment sequences (Figure 4 and Figure 5, respectively) were compared to the transcriptomes of pig, cattle, chicken, salmon, turkey, sheep, goat, cat, and dog (Table 2). A Perl script written by Corteva Agriscience™ (find21ResidueExactMatches.pl) was used to parse the sense and anti-sense fragment sequences into all possible sequentially-overlapping 21-nt subsequences, to parse each transcript into all possible sequentially-overlapping 21-nt subsequences, and then to compare each DvSSJ1-derived 21-nt subsequence to each transcript-derived 21-nt subsequence. For each animal transcriptome, any perfect 21-nt match between a DvSSJ1-derived subsequence and a transcript-derived subsequence would be identified by the script and written to an output file. If no perfect 21-nt matches were identified by the script, then a 'no match found' statement would be written to the output file.

Results and Discussion

No perfect 21-nt matches were identified between the sense or anti-sense DvSSJ1 fragments and any transcript in the pig, cattle, chicken, salmon, turkey, sheep, goat, cat, or dog transcriptome. Consequently, bioinformatics analysis does not indicate the potential for off-target effects of the DvSSJ1 fragment in these farm and companion animals.

Table 2. Transcriptome Dataset Source

Animal	Transcriptome Dataset Webpage
Pig	https://www.ncbi.nlm.nih.gov/genome/84
Cattle	https://www.ncbi.nlm.nih.gov/genome/82
Chicken	https://www.ncbi.nlm.nih.gov/genome/111
Salmon	https://www.ncbi.nlm.nih.gov/genome/369
Turkey	https://www.ncbi.nlm.nih.gov/genome/112
Sheep	https://www.ncbi.nlm.nih.gov/genome/83
Goat	https://www.ncbi.nlm.nih.gov/genome/10731
Cat	https://www.ncbi.nlm.nih.gov/genome/78
Dog	https://www.ncbi.nlm.nih.gov/genome/85

Figure 4. DvSSJ1 Sense Fragment Sequence

ATAATAAGTTCGATTTTTTACGAAAATGACAAGTATCGAGACTGTGGGGACCATTGTCCTGAAATTGCTGAAGTTGG
TGATCAATTTGATATGTCTCATCTTGTACCGAACCGGATATCAAGGCTACTTCTTGGGAGTAGGAGGAACCTGGAAT
CTAAACGAAGAAAAAAATCCCGATGCAGAAATTGTGGCTTCCGGCGTATTCGTAGG

Figure 5. DvSSJ1 Anti-Sense Fragment Sequence

CCTACGAATACGCCGGAAGCCACAATTTCTGCATCGGGATTTTTTTCTTCGTTTAGATTCCAGGTTCCTCCTACTCC
CAAGAAGTAGCCTTGATATCCGGTTCGGTACAAGATGAGACATATCAAATTGATCACCAACTTCAGCAATTTCAGGA
CAATGGTCCCCACAGTCTCGATACTTGTCATTTTCGTAAAAAATCGAACTTATTAT

Appendix D. DvSSJ1 dsRNA Spectrum of Activity Bioassay Materials and Methods

D1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing DvSSJ1_210 double stranded RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. The DvSSJ1_210 dsRNA was produced by Genolution Inc. (Seoul, Korea). Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solutions used to prepare Treatments 2-6 consisted of the test substance diluted in RNase-free water to achieve the concentration in each respective test diet.

Test System

The test system was western corn rootworm (WCR, Diabrotica virgifera virgifera; Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to DvSSJ1_210 dsRNA. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

Multiple independent dose-response bioassays were conducted exposing *WCR* larvae via oral ingestion to the following six treatments:

Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)

Treatment 2: Test Diet (targeting 0.0001 ng DvSSJ1_210 dsRNA per mg diet wet weight)

Treatment 3: Test Diet (targeting 0.001 ng DvSSJ1_210 dsRNA per mg diet wet weight)

Treatment 4: Test Diet (targeting 0.01 ng DvSSJ1_210 dsRNA per mg diet wet weight)

Treatment 5: Test Diet (targeting 0.1 ng DvSSJ1_210 dsRNA per mg diet wet weight)

Treatment 6: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)

Within each bioassay, treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3 replicates from each treatment. Each treatment in each bioassay was fed to a target of 30 WCR individuals. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After each 14-day bioassay, mortality was assessed, and surviving organisms were individually weighed.

For each bioassay, QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in test dosing solutions used to prepare Treatment 6 and the homogeneity of DvSSJ1_210 dsRNA in Treatment 6, from two days of diet preparation. In addition, the stability under bioassay conditions of Treatment 6 (with and without WCR infestation) was verified during one bioassay and was representative of the stability under bioassay conditions of Treatment 6 in each bioassay. The absence of DvSSJ1_210 dsRNA in Treatment 1, using diets from two days of diet preparation, was also verified.

The bioassay acceptability criterion indicated a bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment 1) group.

Bias in the WCR bioassays was controlled through the randomization of treatments within blocks and the use of control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptability criteria. In addition, the use of working stock solutions and bulk standard curves minimized day to day bias.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Dosing solutions for Treatments 1-6 were individually prepared for each bioassay as described in section D.1.a. WCR DvSSJ1 dsRNA Bioassay Analytical Phase. On each day of diet preparation for a bioassay, each dosing solution was mixed with carrier in a 2.51:1 ratio (*i.e.*, 2.51 ml of dosing solution to 1 g of carrier), generating Treatments 1-6.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1 and 6 were collected for characterization as described in section D.1.a. WCR DvSSJ1 dsRNA Bioassay Analytical Phase.

WCR Bioassay

Bioassays were conducted to determine the response of WCR to DvSSJ1_210 dsRNA exposure via oral ingestion. A separate batch of WCR eggs for each bioassay was incubated in an environmental chamber until the eggs hatched. WCR neonates were used in each bioassay within 24 hours of hatching.

On Day 0 of each respective bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One WCR neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 14 days, with the exception of Bioassay 2, where the environmental chamber was incidentally set at 20 °C and 80% relative humidity for the final three days of the bioassay; however, environmental chamber parameters were still within acceptable ranges. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed, with the exception of one organism for which weight was not recorded due to a transfer error. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from statistical analysis.

Statistical Analysis

Statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA) to estimate the median lethal concentration (LC_{50}) of DvSSJ1_210 dsRNA in artificial insect diet for WCR.

Six bioassays were conducted; four met the acceptability criteria and were included in statistical analysis. For each of the four bioassays, a log-logistic regression model was utilized to analyze the dose-response curve data. Let y_{ijk} indicate the mortality status of the k^{th} insect exposed to the j^{th} DvSSJ1_210 dsRNA concentration x_j in the i^{th} bioassay, where i = 1, 2, 5, 6; j = 1, …, 6; k = 1, …, 30; and y_{ijk} = 1 if the insect was dead at the end of the bioassay and y_{ijk} = 0 if the insect was alive. Upon applying Abbott's correction, y_{ijk} was assumed to follow Bernoulli (c_i + (1 - c_i)π_{ij}), where c_i denotes the natural mortality rate in the i^{th} bioassay and π_{ij} denotes the mortality rate caused by exposure to DvSSJ1_210 dsRNA concentration x_j in the i^{th} bioassay. If the observed natural mortality rate was zero in the i^{th} bioassay, then c_i was fixed at zero. The "logit" function was expressed as

$$\text{logit}(\pi_{ij}) = \ln(\frac{\pi_{ij}}{1-\pi_{ij}}),$$

which transformed the parameter π_{ij} on the probability scale into a parameter on the linear predictor scale. The regression model on the linear predictor scale was

$$\text{logit}(\pi_{ij}) = \beta_i \log_{10}(\frac{x_j}{\theta_i}),$$

where β_i denotes the slope and θ_i corresponds to the LC_{50} in the i^{th} bioassay.

SAS PROC NLMIXED was used to fit the log-logistic regression model for each bioassay. Model parameter estimates and their standard errors (SE) were obtained using the maximum likelihood method.

A random-effects meta-analysis approach (Normand, 1999) was utilized in SAS PROC MIXED to derive the estimate of the overall mean LC_{50}, $\hat{\theta}$, across the bioassay:

$$\hat{\theta} = \frac{\sum_{i=1}^{4} W_i \hat{\theta}_i}{W_i} \; with \; W_i = \frac{1}{\hat{S}_i^{\,2} + \hat{\tau}^2}$$

where $\hat{\theta}_i$ represents the estimate of θ_i for the i^{th} bioassay, $\hat{s}_i$ represents the estimate of SE for $\hat{\theta}_i$, and $\hat{\tau}^2$ represents the estimate of the variation of LC_{50} among bioassays. The estimated SE of $\hat{\theta}$ was $(\sum_{i=1}^{4} W_i)^{-\frac{1}{2}}$.

The LC_{50} estimate, SE, and 95% confidence interval for each bioassay as well as the overall means are reported in Table 3.

Results and Discussion

The bioassay acceptability criterion for the WCR bioassays indicated a bioassay could be terminated and repeated if the combined number of dead and missing organisms exceeded 30% in the bioassay control diet (Treatment 1). Bioassays 3 and 4 did not meet the acceptability criterion and were terminated. Bioassays 1, 2, 5, and 6 met the acceptability criterion and those results are reported. Details regarding the terminated bioassays will not be reported but will be maintained in the study records.

The LC_{50} point estimate, SE, and 95% confidence interval for each individual bioassay, as well as the overall means derived by meta-analysis across the four bioassays, are reported in Table 3. The estimated overall mean LC_{50} for WCR was 0.036 ng DvSSJ1_210 dsRNA per mg diet wet weight with a 95% confidence interval of 0.0066 - 0.065 ng DvSSJ1_210 dsRNA per mg diet (Table 3).

Observed WCR mortality data are summarized in Table 4. Statistically fitted dose-response curves for mortality versus DvSSJ1_210 dsRNA concentration for each WCR bioassay are shown in Figure 6.

For each bioassay, QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in test dosing solutions used to prepare Treatment 6 and the absence of DvSSJ1_210 dsRNA in Treatment 1, from two days of diet preparation (Table 5). The homogeneity of DvSSJ1_210 dsRNA in Treatment 6 was also verified twice during each bioassay (Table 5). Homogeneity samples were within the acceptable range. Therefore, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range is 0.283. In addition, the stability under bioassay conditions of Treatment 6 was verified for four days, both with and without WCR infestation (Table 6).

A summary of WCR larval weight data is provided in Table 7.

Table 3. Median Lethal Concentration of DvSSJ1_210 dsRNA for WCR

Bioassay	LC_{50}		
	Point Estimate	**Standard Error**	**95% Confidence Interval**
1	0.045	0.019	0.0064 - 0.083
2	0.042	0.027	0 - 0.096
5	0.084	0.033	0.019 - 0.15
6	0.011	0.0077	0 - 0.026
Overall Mean[a]	0.036	0.015	0.0066 - 0.065

Note: Median lethal concentration (LC_{50}) is expressed in ng DvSSJ1_210 dsRNA per mg diet wet weight

[a] The overall mean values as determined with the statistical analysis.

The estimated overall mean LC_{50} for WCR was 0.036 ng DvSSJ1_210 dsRNA per mg diet wet weight with a 95% confidence interval of 0.0066 - 0.065 ng DvSSJ1_210 dsRNA per mg diet.

Table 4. Summary Analysis of WCR Larval Mortality

Bioassay	Treatment	Treatment Description[a]	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
1	1	Bioassay Control Diet	29	6	20.7
	2	Test Diet (0.0001 ng DvSSJ1/mg)	28	2	7.14
	3	Test Diet (0.001 ng DvSSJ1/mg)	28	2	7.14
	4	Test Diet (0.01 ng DvSSJ1/mg)	26	11	42.3
	5	Test Diet (0.1 ng DvSSJ1/mg)	26	17	65.4
	6	Test Diet (1 ng DvSSJ1/mg)	29	27	93.1
2	1	Bioassay Control Diet	28	7	25.0
	2	Test Diet (0.0001 ng DvSSJ1/mg)	27	11	40.7
	3	Test Diet (0.001 ng DvSSJ1mg)	25	8	32.0
	4	Test Diet (0.01 ng DvSSJ1 /mg)	27	10	37.0
	5	Test Diet (0.1 ng DvSSJ1/mg)	30	27	90.0
	6	Test Diet (1 ng DvSSJ1/mg)	28	28	100
5	1	Bioassay Control Diet	28	6	21.4
	2	Test Diet (0.0001 ng DvSSJ1/mg)	29	9	31.0
	3	Test Diet (0.001 ng DvSSJ1/mg)	30	7	23.3
	4	Test Diet (0.01 ng DvSSJ1/mg)	30	9	30.0
	5	Test Diet (0.1 ng DvSSJ1/mg)	30	20	66.7
	6	Test Diet (1 ng DvSSJ1/mg)	29	28	96.6
6	1	Bioassay Control Diet	29	5	17.2
	2	Test Diet (0.0001 ng DvSSJ1/mg)	28	10	35.7
	3	Test Diet (0.001 ng DvSSJ1/mg)	28	5	17.9
	4	Test Diet (0.01 ng DvSSJ1/mg)	28	20	71.4
	5	Test Diet (0.1 ng DvSSJ1 mg)	29	23	79.3
	6	Test Diet (1 ng DvSSJ1/mg)	27	25	92.6

[a] Targeted DvSSJ1_210 dsRNA concentrations in Treatments 2-6 are based on diet wet weight.

Table 5. Verification of DvSSJ1_210 dsRNA Concentration in Treatment 6 Test Dosing Solutions, Homogeneity of Treatment 6, and Absence of DvSSJ1_210 dsRNA in Treatment 1 for the WCR bioassay.

Bioassay	Concentration[a] Treatment 6 Test Dosing Solution				Homogeneity[b] Treatment 6				Absence of DvSSJ1_210 dsRNA Treatment 1			
	Day	Result (%)	Day	Result (%)	Day	Result	Day	Result	Day	Result	Day	Result
1	0	84	4	94	0	Verified	4	Verified	0	Absent	4	Absent
2	4	89	7	99	4	Verified	7	Verified	4	Absent	7	Absent
5	7	99	10	111	7	Verified	10	Verified	7	Absent	10	Absent
6	4	118	7	118	4	Verified	7	Verified	4	Absent	7	Absent

[a] Concentrations within 70-130% of the expected value were considered verified.
[b] Homogeneity was considered verified for a given bioassay and diet preparation day if the value of each sample was within 70-130% of the mean value of all samples analyzed for that bioassay and diet preparation day.

Table 6. Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 6 for the WCR bioassay

Day and Condition	Average % of Day 0 Mean[a]
Day 0 (Not Infested)	NA
Day 1 (Not Infested)	99
Day 2 (Not Infested)	95
Day 3 (Not Infested)	92
Day 4 (Not Infested)	90
Day 4 (Infested)	91

Note: Not infested/infested: Not infested diet samples were placed in bioassay plates that were not infested with WCR prior to analysis; infested diet samples were collected from bioassay plates infested with WCR prior to analysis. Not applicable (NA).
[a] Diet samples were considered stable if the average percentage for each sampling time point and condition was ≥ 70% of the Day 0 mean. Stability under bioassay conditions of Treatment 6 in one bioassay was considered representative of stability under bioassay conditions of Treatment 6 in each of the bioassays.

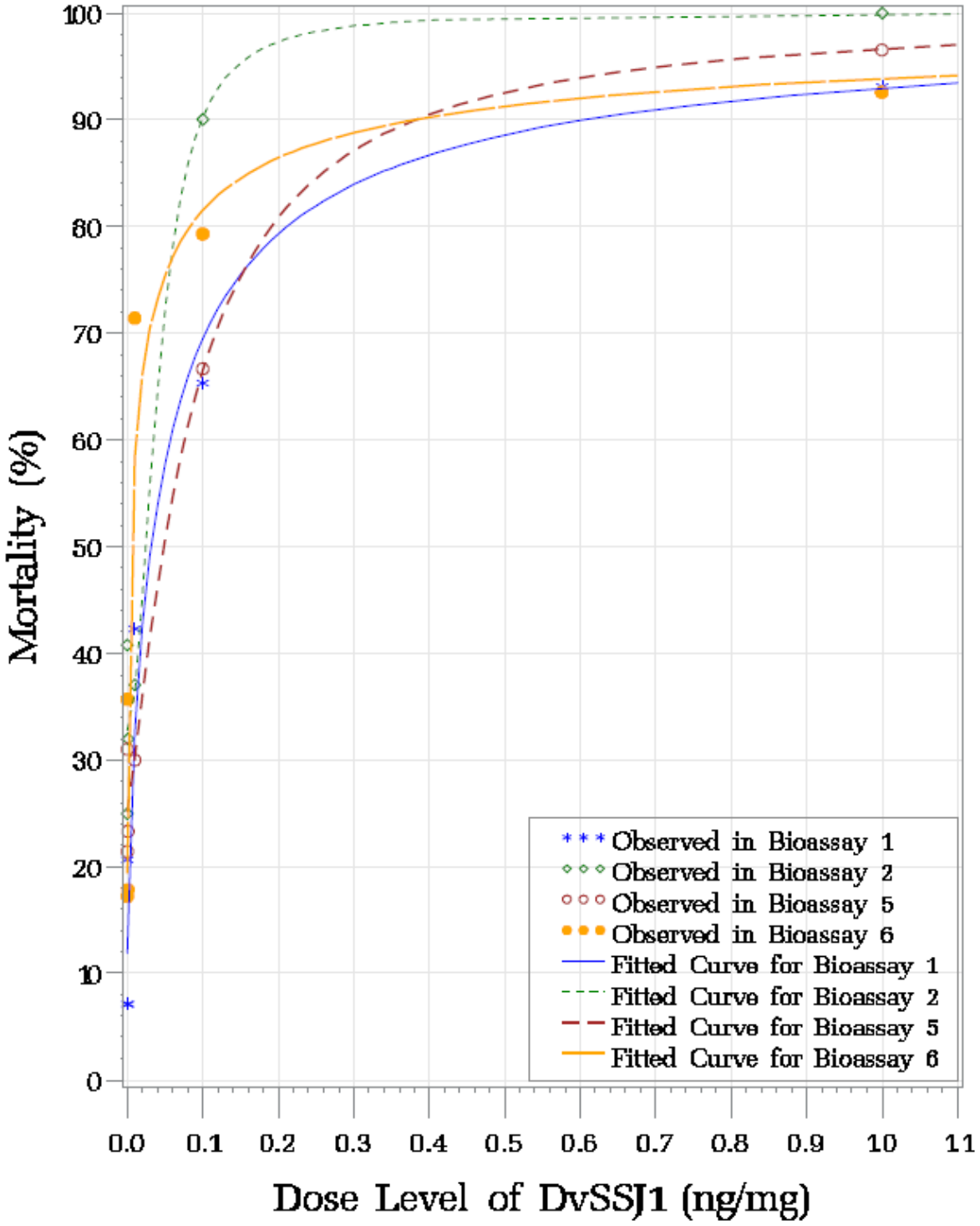


Figure 6. Observed Mortality Rates and the Estimated Dose Response Curve for Each *WCR* Bioassay

D.1.a. WCR DvSSJ1 dsRNA Bioassay Analytical Phase

The following dosing solutions were prepared for the *Diabrotica virgifera virgifera (*WCR) bioassays:

- Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water
- Test dosing solutions used to prepare Treatments 2-6 consisting of DvSSJ1_210 double-stranded RNA (dsRNA) test substance diluted in RNase-free water to achieve the concentrations in the test diets

For each bioassay, QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in test dosing solutions used to prepare Treatment 6 and the homogeneity of DvSSJ1_210 dsRNA in Treatment 6, from two days of diet preparation. In addition, the stability under bioassay conditions of Treatment 6 (with and without WCR infestation) was verified during one bioassay and was representative of the stability under bioassay conditions of Treatment 6 in each bioassay. The absence of DvSSJ1_210 dsRNA in Treatment 1, using diets from two days of diet preparation, was also verified. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Test substance was diluted in RNase-free water and stored frozen (-80 °C freezer unit) to create DvSSJ1_210 dsRNA working stock solutions for the bioassays. On each day of diet preparation, working stock solution (either freshly prepared or thawed at room temperature from frozen storage) was serially diluted in RNase-free water to prepare the dosing solutions for Treatments 2-5 and used directly to prepare the dosing solution for Treatment 6. The DvSSJ1_210 dsRNA concentrations in the test dosing solutions ranged from 0.14-1400 ng/ml to achieve the desired concentrations in Treatments 2-6. The bioassay control dosing solution for each bioassay consisted of RNase-free water. Dosing solutions were prepared and maintained at room temperature. Twice during each bioassay, samples of Treatment 6 dosing solution were collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:14 dilution (10 µl test dosing solution to 130 µl QHS) and stored frozen (-80 °C freezer unit) until analysis, if applicable, for verification of the concentration of DvSSJ1_210 dsRNA.

Characterization of Diets

Sample Collection and Stability Under Bioassay Conditions

During the process of diet distribution for the WCR bioassays, samples of Treatment 1 and Treatment 6 (~300 mg each) were collected into tubes twice during each bioassay. For verification of the absence of DvSSJ1_210 dsRNA in Treatment 1, five samples were collected on two diet preparation days. For verification of homogeneity in Treatment 6, five samples each were collected from the beginning, middle, and end of diet distribution for two diet preparation days in each bioassay. Samples were stored frozen (-80 °C freezer unit) until analysis.

In addition, samples (~300 mg each) were collected for verification of stability under bioassay conditions of Treatment 6. For the Day 0 assessment, five samples were collected into tubes during Day 0 diet distribution for the WCR bioassay and stored frozen (-80 °C freezer unit) until analysis. For the Days 1-4 assessment, samples (~300 mg each) were distributed to bioassay plates (five samples per plate; 20 total) on Day 0 and placed under bioassay conditions using the same methods and conditions used in the WCR bioassay, except they were not infested with larvae.

For each day of stability analysis (Days 1-4), one bioassay plate containing five samples of Treatment 6 was removed from bioassay conditions and stored frozen (-80 °C freezer unit) until analysis. In addition, Treatment 6 that had been fed to WCR was collected following transfer of organisms during the Day 4 refeed and stored frozen (-80 °C freezer unit) until analysis for assessment of stability under bioassay conditions with infestation.

Preparation of Sample Homogenates

Collected diet samples (nine Treatment 6 homogeneity samples three each from beginning, middle, end and three samples of Treatment 1 from each of two diet preparation days, as well as three samples of Day 0 bioassay stability) were removed from the freezer in preparation for extraction and analysis. Treatment 6 samples (three for each of Days 1-4 without infestation and three from Day 4 with infestation) frozen in bioassay plates were removed from bioassay plates and placed into tubes. QHS (3000 µl) was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were transferred to individual tubes and centrifuged again. The supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized a magnetic bead-based assay kit to quantify the concentration of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), thawed and warmed, and then diluted as necessary in QHS. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture

Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

Standard curves were prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentrations of DvSSJ1_210 dsRNA in the Treatment 6 dosing solutions were interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability under bioassay conditions), the background-corrected Median Fluorescence Intensity (MFI) was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentrations in the Treatment 6 Test Dosing Solutions

The concentration of DvSSJ1_210 dsRNA in test dosing solutions was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 6

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 6 samples analyzed for a given bioassay and diet preparation day.

The acceptable range for each diet preparation day was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 6

Stability was calculated for each time point and condition (with and without WCR infestation) as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling day and condition (Days 1 – 4 without WCR and Day 4 with WCR). Stability was considered verified if the average percentage for a sampling time point and condition was greater than or equal to 70% of the Day 0 mean.

Summary of WCR Larval Weight Data

Table 7. Summary of WCR Larval Weight Data

Bioassay	Treatment	Treatment Description	Number of Surviving Organisms	Weight (mg)		
				Mean	Standard Deviation	Range
1	1	Bioassay Control Diet	23	1.71	0.749	0.5 - 2.8
	2	Test Diet (0.0001 ng DvSSJ1/mg)	26	1.81	0.740	0.6 - 2.9
	3	Test Diet (0.001 ng DvSSJ1/mg)	26	1.90	0.771	0.5 - 3.2
	4	Test Diet (0.01 ng DvSSJ1/mg)	15	1.11	0.746	0.3 - 2.6
	5	Test Diet (0.1 ng DvSSJ1/mg)	9	0.633	0.520	0.2 - 1.9
	6	Test Diet (1 ng DvSSJ1/mg)	2	0.550	0.354	0.3 - 0.8
2	1	Bioassay Control Diet	21	1.59	0.751	0.3 - 2.9
	2	Test Diet (0.0001 ng DvSSJ1/mg)	16	1.99	0.599	0.7 - 3.0
	3	Test Diet (0.001 ng DvSSJ1/mg)	17	1.36	0.668	0.3 - 2.7
	4	Test Diet (0.01 ng DvSSJ1/mg)	17	1.08	0.768	0.3 - 2.8
	5	Test Diet (0.1 ng DvSSJ1/mg)	3	0.400	0.173	0.3 - 0.6
	6	Test Diet (1 ng DvSSJ1/mg)	0	NA	NA	NA
5	1	Bioassay Control Diet	22	1.01	0.638	0.3 - 2.5
	2	Test Diet (0.0001 ng DvSSJ1/mg)	20	1.66	0.703	0.3 - 2.9
	3	Test Diet (0.001 ng DvSSJ1/mg)	23	1.21	0.666	0.3 - 2.5
	4	Test Diet (0.01 ng DvSSJ1/mg)	21[a]	0.655	0.394	0.2 - 1.8
	5	Test Diet (0.1 ng DvSSJ1/mg)	10	0.410	0.260	0.2 - 1.1
	6	Test Diet (1 ng DvSSJ1/mg)	1	0.200	NC[b]	NC
6	1	Bioassay Control Diet	24	1.58	0.816	0.4 - 3.1
	2	Test Diet (0.0001 ng DvSSJ1/mg)	18	0.967	0.778	0.1 - 2.8
	3	Test Diet (0.001 ng DvSSJ1/mg)	23	1.20	0.577	0.3 - 2.4
	4	Test Diet (0.01 ng DvSSJ1/mg)	8	0.588	0.479	0.1 - 1.6
	5	Test Diet (0.1 ng DvSSJ1/mg)	6	0.367	0.186	0.2 - 0.7
	6	Test Diet (1 ng DvSSJ1/mg)	2	0.200	0.141	0.1 - 0.3

Note: Targeted DvSSJ1_210 dsRNA concentrations in Treatments 2-6 are based on diet wet weight. Not applicable (NA); there were no surviving WCR in Bioassay 2, Treatment 6.

[a] Weight was not recorded for one organism due to a transfer error.

[b] The reported mean is the weight value of the one surviving organism and the standard deviation and range are not calculated (NC).

D2. Evaluation of the Survival and Weight of Southern Corn Rootworm Fed Artificial Diets Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted primarily of southern corn rootworm diet suspended in agar.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solutions used to prepare Treatments 2-6 consisted of the test substance diluted in RNase-free water to achieve the concentration in each respective test diet.

Test System

The test system was *Diabrotica undecimpunctata* (southern corn rootworm; Coleoptera: Chrysomelidae SCR).

SCR was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. *SCR* eggs were obtained from Crop Characteristics Inc. (Farmington, MN, USA) and identity was recorded by study personnel.

Experimental Design

SCR larvae were exposed via oral ingestion to one of the following six treatments:

Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)

Treatment 2: Test Diet (targeting 0.001 µg DvSSJ1_210 dsRNA per ml diet wet weight)

Treatment 3: Test Diet (targeting 0.01 µg DvSSJ1_210 dsRNA per ml diet wet weight)

Treatment 4: Test Diet (targeting 0.1 µg DvSSJ1_210 dsRNA per ml diet wet weight)

Treatment 5: Test Diet (targeting 1 µg DvSSJ1_210 dsRNA per ml diet wet weight)

Treatment 6: Test Diet (targeting 10 µg DvSSJ1_210 dsRNA per ml diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3 replicates from each treatment. Each treatment was fed to a target of 30 *SCR* individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 7. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criterion indicated the bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment 1) group.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in test dosing solutions used to prepare Treatment 5 and the homogeneity and the stability under bioassay conditions of DvSSJ1_210 dsRNA in a test diet (Treatment 5). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified.

Bias in the *SCR* bioassay was controlled through the randomization of treatments within blocks. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. In addition, the use of a working stock solution and facility standard curve preparation minimized day to day bias.

Diet Generation

The DvSSJ1_210 dsRNA working stock solution and dosing solutions for Treatments 1-6 were prepared as described in section D2.a. SCR DvSSJ1 dsRNA Bioassay Analytical Phase On each day of diet preparation, each dosing solution was mixed with carrier to generate Treatments 1-6.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1 and 5 were collected for characterization of diets as described in the D2.a. SCR DvSSJ1 dsRNA Bioassay Analytical Phase section below.

***SCR* Bioassay**

SCR eggs were incubated in an environmental chamber until the eggs hatched.

SCR neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 1 ml of freshly prepared agar-suspended diet was dispensed into wells of the bioassay plates. The diet in each well was allowed to cool and solidify then was lightly scored.

One *SCR* neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark for a total of 14 days. On Day 7, new bioassay plates were prepared with fresh diet as described for Day 0, Living *SCR* larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4

The response variables of interest were mortality and weight. Statistical comparisons were made between *SCR* fed diet containing DvSSJ1_210 dsRNA (Treatments 2, 3, 4, 5, and 6) and the bioassay control diet (Treatment 1) for each response variable

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of SCR fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis tests were

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The distributions of weight were evaluated for each treatment. The data were not normally distributed; therefore, non-parametric two-sample tests were conducted separately to examine if exposure to DvSSJ1_210 dsRNA in each test diet caused growth inhibition compared to exposure to the bioassay control diet. The corresponding hypothesis tests were

$$H_0 : F_T(x) = F_c(x) \ \ vs. \ H_a : F_T(x) < F_c(x)$$

Where $F_T(x)$ is the cumulative distribution of the weight of *SCR* fed the artificial insect diet containing DvSSJ1_210 dsRNA, and $F_c(x)$ is the cumulative distribution of the weight of individuals fed the bioassay control diet. Therefore, significance would indicate that insects fed the diets containing DvSSJ1_210 dsRNA would have a greater probability to weigh less than those fed the control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Results and Discussion

The *SCR* bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 30%, as shown in **Table 8**.

The mortality of *SCR* fed the test diets containing 0.001 and 0.01 µg DvSSJ1_210 dsRNA per ml diet (Treatments 2 and 3; 13.3 and 10.0%; P-values = 0.500 and 0.6646, respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1; 10.0%), as shown in **Table 8**. The mortality of *SCR* fed the test diets containing 0.1, 1, and 10 µg DvSSJ1_210 dsRNA per ml diet (Treatments 4, 5, and 6; 33.3, 70.0, and 80.0%; P-values = 0.0287, <0.0001, and <0.0001, respectively) was significantly greater than those fed the bioassay control diet, as shown in **Table 8**.

SCR fed Treatment 2 (median 5.05 mg; Wilcoxon test P-value = 0.3029) did not have significantly greater probability to weigh less than those fed Treatment 1 (median 5.1 mg), while the Siegel-Tukey test showed significant evidence that the scales of the population differed, as shown in **Table 9**. *SCR* fed the test diets containing 0.01, 0.1, 1, and 10 µg DvSSJ1_210 dsRNA per ml diet (Treatments 3-6; median 2.7, 1.2, 2.5, and 1.6 mg; Wilcoxon test P-values = 0.0031, <0.0001, 0.0163, and 0.0062, respectively) had significantly greater probability to weigh less than those fed Treatment 1, as shown in **Table 9**. In addition, the

Siegel-Tukey test (P-values = 0.9862, 0.7354, 0.8693, and 0.4589 for Treatments 3-6, respectively) did not show significant evidence that the scales of the populations differed, as shown in **Table 9**.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the Day 0 Treatment 5 test dosing solution (122% of expected value). The concentration of the Day 7 Treatment 5 test dosing solution was 139% of expected, which was above the acceptance criteria of 70-130%; however, Treatment 5 diets prepared on Days 0 and 7 had median fluorescence intensities (MFIs) that were nearly the same. In addition, both sets of dosing solutions were prepared from the same working stock solution and the result for the Treatment 5 dosing solution prepared on Day 7 is only 14% different than the result for the dosing solution prepared on Day 0. The Day 7 result is likely attributed to an analytical artifact related only to the sample collected and diluted in QHS on the day of dosing solution preparation. All samples for the study were analyzed on the same plate. The impact of the dosing solution interpolating just above the acceptance criteria was determined to have no impact, especially considering the 10-fold differences in treatment concentrations provided to insects.

QuantiGene analysis verified the homogeneity of DvSSJ1_210 dsRNA in the Day 0 Treatment 5 diet. Homogeneity assessment for one day of diet preparation is considered representative of both days of diet preparation. The absence of DvSSJ1_210 dsRNA in Treatment 1 was also verified. The stability of DvSSJ1_210 dsRNA in Treatment 5 stored under bioassay conditions was confirmed over seven days.

Conclusion

The results demonstrated the mortality of *SCR* fed the test diets containing 0.001 and 0.01 µg DvSSJ1_210 dsRNA per ml diet (Treatments 2 and 3; 13.3% and 10.0%, respectively) was not significantly greater than the mortality of *SCR* fed the bioassay control diet (Treatment 1; 10.0%). Statistically significant differences were observed in mortality between *SCR* fed the test diets containing 0.1, 1, and 10 µg DvSSJ1_210 dsRNA per ml diet (Treatments 4-6; 33.3%, 70.0%, and 80.0%, respectively) and those fed Treatment 1. *SCR* fed Treatments 3-6 had a significantly greater probability of reduced weight as compared to those fed Treatment 1, with no significant difference in probability observed for Treatment 2.

Table 8. Summary Analysis of SCR Mortality Results

Treatment	Treatment Description	Treatment Dose (µg DvSSJ1/ml)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	30	3	10.0	--
2	Test Diet	0.001	30	4	13.3	0.5000
3	Test Diet	0.01	30	3	10.0	0.6646
4	Test Diet	0.1	30	10	33.3	0.0287[a]
5	Test Diet	1	30	21	70.0	<0.0001[a]
6	Test Diet	10	30	24	80.0	<0.0001[a]

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatments 2-6 was based on diet wet weight.
[a] A statistically significant difference (P-value < 0.05) was observed.

Table 9. Summary Analysis of SCR Weight Results

Treatment	Treatment Description	Treatment Dose (µg DvSSJ1/ml)	Number of Surviving Organisms	Mean Standard Deviation (mg)	Median Weight (mg)	Range (mg)	Wilcoxon Test P-Value	Siegel Tukey Test P-Value
1	Bioassay Control Diet	0	27	7.94 ± 6.34	5.1	0.4 - 22.6	--	--
2	Test Diet	0.001	26	8.01 ± 7.42	5.05	0.4 - 20.8	0.3029	0.0494[a]
3	Test Diet	0.01	27	3.69 ± 3.55	2.7	0.4 - 15.0	0.0031[a]	0.9862
4	Test Diet	0.1	20	1.49 ± 1.07	1.2	0.3 - 3.7	<0.0001[a]	0.7354
5	Test Diet	1	9	3.30 ± 2.39	2.5	0.2 - 8.2	0.0163[a]	0.8693
6	Test Diet	10	6	2.03 ± 1.81	1.6	0.4 - 4.6	0.0062[a]	0.4589

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatments 2-6 was based on diet wet weight.
[a] A statistically significant difference (P-value < 0.05) was observed.

Table 10. Verification of DvSSJ1_210 dsRNA Concentration in Test Dosing Solution, Homogeneity in Test Diet, and Absence in SCR Bioassay Control Diet

Analysis	Diet Preparation Day	Result
Test Dosing Solution Concentration Verification	0	122% of expected[a]
	7	139% of expected[a]
Homogeneity in Test Diet (Treatment 5)	0	Verified[b]
Absence in Bioassay Control Diet (Treatment 1)	0	Verified[c]

[a] Concentrations within 70-130% of the expected value were considered verified. The Day 7 result is likely attributed to an analytical artifact.
[b] Homogeneity was considered verified if the value of each sample was within 70-130% of the mean value of all samples analyzed.
[c] Absence of DvSSJ1_210 dsRNA in Treatment 1 was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

Table 11. Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in SCR Test Diet

Bioassay Stability Day (Treatment 5)	Average % of Day 0 Mean[a]
Day 0	NA
Day 2	89
Day 5	85
Day 7	86

Note: Not applicable (NA).

[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

D2.a. SCR DvSSJ1 dsRNA Bioassay Analytical Phase

The following dosing solutions were prepared for the SCR (*SCR*) bioassay:

- Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water
- Test dosing solutions used to prepare Treatment 2-6 consisting of test substance diluted in RNase-free water to achieve the concentrations in the test diets

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in test dosing solutions used to prepare Treatment 5, as well as the homogeneity and stability under bioassay conditions of DvSSJ1_210 dsRNA in a test diet (Treatment 5). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Test substance was diluted in RNase-free water to create a DvSSJ1_210 dsRNA working stock solution. On each day of diet preparation, working stock solution (either freshly prepared or thawed at room temperature from frozen storage at -80 °C) was serially diluted in RNase-free water to prepare the dosing solutions for Treatments 2-5 and used directly to prepare the dosing solution for Treatment 6. The DvSSJ1_210 dsRNA concentrations in the test dosing solutions ranged from 0.0034-34 µg/ml to achieve the desired concentrations in Treatments 2-6. The bioassay control dosing solution consisted of RNase-free water. Dosing solutions were prepared and maintained at room temperature.

Sample Collection

Twice during the bioassay, samples of Treatment 5 dosing solution were collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:34 dilution factor (10 µl test dosing solution to 330 µl QHS) and stored frozen (-80 °C freezer unit) until analysis, if applicable, for verification of the concentration of DvSSJ1_210 dsRNA. During the process of diet distribution for the *SCR* bioassay, samples (~1 ml each) of Treatments 1 and 5 were collected into tubes as described in **Table 12**. Samples were stored frozen if applicable (-80 °C freezer unit) until analysis.

Table 12. DvSSJ1_210bp dsRNA Dosing Solutions for the *SCR*Bioassay

Treatment	Diet Preparation Day	Number of Samples	Analysis
1	0	5	Verify absence of DvSSJ1_210 dsRNA
5	0[a]	5 Beginning 5 Middle 5 End	Homogeneity of DvSSJ1_210 dsRNA
	7	20[b]	Stability under bioassay conditions

[a] Homogeneity assessment for one day of diet preparation is considered representative of both days of diet preparation.
[b] Five samples were collected into tubes; the remaining samples were distributed to bioassay plates and placed under bioassay conditions.

Stability under Bioassay Conditions

Samples were collected during the process of diet distribution for verification of stability of DvSSJ1_210 dsRNA in Treatment 5 under bioassay conditions. For the Day 0 assessment, five samples were collected into tubes and stored frozen (-80 °C freezer unit) until analysis. For the Days 2, 5, and 7 assessments, samples were randomly distributed to bioassay plates (five samples per plate; 15 total) and placed under bioassay conditions using the same methods and conditions used in the *SCR* bioassay, except they were not infested with larvae. Day 0 samples for bioassay stability were not placed under bioassay conditions. For each day of stability analysis (Days 2, 5, and 7), one bioassay plate containing five samples of Treatment 5 was removed from bioassay conditions, the diets transferred to tubes, and then stored frozen (-80 °C freezer unit) until analysis.

Preparation of Sample Homogenates

Diet samples were removed from the freezer in preparation for extraction and analysis (three each from the beginning, middle, and end of diet distribution for homogeneity, three for Treatment 1 verification of the absence of DvSSJ1_210 dsRNA, and three for each day of bioassay stability).

QHS (4 ml) was added to each tube containing diet and the samples placed in a heat block set at 100°C for approximately 10 minutes. Samples were vortexed until no clumps were visible. A sub-sample (200 µl) was collected into a new tube, 200 µl of QHS was added, and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were transferred to individual tubes and centrifuged again. The supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), thawed and warmed, and then diluted as necessary in QHS. Treatment 1 samples were loaded to the plate undiluted. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the Treatment 5 dosing solutions was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability under bioassay conditions), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 5 Test Dosing Solutions

The concentration of DvSSJ1_210 dsRNA in test dosing solution was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 5

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 5 samples analyzed.

The acceptable range was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in the verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Verification of Absence of DvSSJ1_210 dsRNA in Treatment 1

Absence of DvSSJ1_210 dsRNA in Treatment 1 samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean MFI of lowest Standard Curve Point – 10%)

Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 5

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling day (Days 2, 5, and 7). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

D3. Evaluation of the Survival and Weight of Colorado Potato Beetle Fed an Artificial Diet Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted primarily of Stonefly Heliothis diet.

Positive Control

The positive control consisted of cryolite (AlF_6Na_3).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solution used to prepare Treatment 2 consisted of the test substance diluted in RNase-free water to achieve the concentration in the test diet.

Test System

The test system was *Leptinotarsa decemlineata* (Colorado potato beetle; Coleoptera: Chrysomelidae). *CPB* was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. *CPB* eggs were obtained from French Agricultural Research, Inc. (Lamberton, MN, USA) and identity was recorded by study personnel.

Experimental Design

CPB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 5000 ng cryolite per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained 3 replicates from each

treatment. Each treatment was fed to a target of 30 *CPB*. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed every other day and missing and dead organisms were recorded. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity and stability under bioassay conditions of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

Bias in the *CPB* bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. In addition, the use of bulk dosing solutions and bulk standard curve preparation minimized day to day bias.

Diet Generation

Bulk dosing solutions for Treatments 1 and 2 were prepared and maintained as described in Appendix A. On each day of diet preparation, each dosing solution was mixed with carrier in a 2.8:1 ratio (*i.e.*, 2.8 ml of dosing solution to 1 g of carrier). Treatment 3 was prepared by mixing cryolite with carrier to a nominal concentration of 5000 ng per mg carrier dry weight and then combining with RNase-free water in a 2.8:1 ratio.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1 and 2 were collected for characterization of diets as described in section D3.a. Colorado Potato Beetle DvSSJ1 dsRNA Bioassay Analytical Phase. In addition, a portion each of Treatment 1 and Treatment 2 was collected for use in diets for the sensitive insect bioassay as described in sectionD3.b. Colorado Potato Beetle DvSSJ1 dsRNA Sensitive Insect Bioassay.

The positive control diet (Treatment 3) was not characterized.

CPB Bioassay

CPB eggs were incubated in an environmental chamber until the eggs hatched. *CPB* neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One CPB neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark for a total of 14 days. Every other day, new bioassay plates were prepared with fresh diet as described for Day 0, with the exception that on Days 10 and 12, 600 µl was dispensed per well. The additional diet was needed to accommodate the increased nutritional needs of the growing organisms. Living CPB larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; wells containing more than one organism were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparison was made between CPB fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

CPB fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2; therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition, *i.e.*, lower weight of *CPB* fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was:

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatment 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Demonstration of DvSSJ1_210 dsRNA Activity in the *CPB* Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay. WCR was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA used in Treatment 2 in the *CPB* bioassay. Details regarding the sensitive insect bioassay are provided in section D3.b. Colorado Potato Beetle DvSSJ1 dsRNA Sensitive Insect Bioassay.

Results and Discussion

The *CPB* bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 13.

The CPB bioassay was run three times. In the initial two runs, the bioassays did not meet the acceptability criteria as the number of dead and missing larvae in Treatment 1 exceeded 20%. The third run of the bioassay met the acceptability criteria; therefore, these results are included in this appendix. Each CPB bioassay was run using freshly prepared dosing solutions and diets and a sensitive insect bioassay was conducted. Data generated for the first two runs of the CPB and WCR bioassays as well as diet characterization results for the CPB bioassays were not included in this report, but were retained with the study records.

The mortality of CPB fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 3.33%; P-value = 0.7627) was not significantly greater than those fed the bioassay control diet (Treatment 1; 3.45%), as shown in Table 13.

The mean weight of CPB fed Treatment 2 (153 mg; P-value = 0.9471) was not significantly less than the mean weight of those fed Treatment 1 (136 mg), as shown in Table 14.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the Treatment 2 test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in Treatment 2 (Table 16). Homogeneity verification on two days of diet preparation is considered representative of all days of diet preparation. The stability of DvSSJ1_210 dsRNA in Treatment 2 stored under bioassay conditions was verified over two days (Table 17).

The absence of DvSSJ1_210 dsRNA in Treatment 1 was assessed in three collected samples for each of Days 0 and 12. For Day 0, the absence of DvSSJ1_210 dsRNA was verified in all analyzed samples. For Day 12, the absence of DvSSJ1_210 dsRNA was verified in one sample but trace amounts were found in the other two samples (Table 16). Those samples were reanalyzed along with two additional Day 12 samples. Upon re-analysis, the finding was confirmed for the original two samples and a trace amount of DvSSJ1_210 dsRNA was also detected in one of the two new samples. Treatment 1 samples were analyzed undiluted while Treatment 2 samples were diluted 1:16,000 for analysis. If diluted to the same level as Treatment 2 samples, the DvSSJ1_210 dsRNA signal in the affected Treatment 1 samples would not have been detectable and it is estimated that the signal would have been at least 3,000 times less than in Treatment 2 based on the results of each Treatment 1 sample. Although there were trace amounts of DvSSJ1_210 dsRNA detected in select samples of the bioassay control diet, results for *CPB* mortality and weight in this study compared to historical data indicate no effect on the bioassay control diet group response and no impact on this study.

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 15. The WCR bioassay met the acceptability criterion. The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 of the CPB bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B) when compared to the bioassay control diet (Treatment A).

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of CPB.

Table 13. Summary Analysis of CPB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	29 [a]	1	3.45	--
2	Test Diet	1	30	1	3.33	0.7627
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Wells that contained more than one organism were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 5000 ng cryolite per mg diet dry weight.

Table 14. Summary Analysis of CPB DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	136 (118 - 154)	7.2 - 217.0	--
2	Test Diet	1	29	153 (141 - 165)	54.7 - 200.8	0.9471
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving *Leptinotarsa decemlineata* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 5000 ng cryolite per mg diet dry weight.

Table 15. Summary of CPB DvSSJ1 dsRNA Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	30	26.7	22	1.62 ± 0.804	0.1 - 3.0
B	Test Diet	0.05	29[a]	72.4	8	0.363 ± 0.213	0.1 - 0.8

Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the CPB bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 16. Assessment of DvSSJ1_210 dsRNA Concentration in Test Dosing Solution, Homogeneity in Test Diet, and Absence in Bioassay Control Diet for the CPB Bioassay

Analysis	Diet Preparation Day	Result
Test Dosing Solution Concentration Verification	0	Verified[a]
	After final use	Verified[a]
Homogeneity in Test Diet (Treatment 2)	0	Verified[b]
	12	Verified[b]
Absence in Bioassay Control Diet (Treatment 1)	0	Verified[c]
	12	Trace[d]

[a] The test dosing solution concentration was 122% and 110% of the expected DvSSJ1_210 dsRNA concentration for the Day 0 and after final use samples, respectively. Concentrations within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified for a given Treatment 2 diet preparation if the value of each sample was within 70-130% of the mean value of all samples analyzed for that diet preparation. Three samples each from the beginning, middle, and end of diet distribution were analyzed for each day.

[c] Absence of DvSSJ1_210 dsRNA in Treatment 1 was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

[d] Trace amounts of DvSSJ1_210 dsRNA were detected in three out of five bioassay control samples. The quantity detected is estimated to be over 3,000-fold less concentrated than in the test diet.

Table 17. Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in CPB Test Diet

Bioassay Stability Day	Average % of Day 0 Mean[a]
Day 0	NA
Day 1	114
Day 2	113

Note: NA (not applicable).

[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

D3.a. Colorado Potato Beetle DvSSJ1 dsRNA Bioassay Analytical Phase

The following dosing solutions were prepared for the CPB bioassay:

- Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water
- Test dosing solution used to prepare Treatment 2 consisting of test substance diluted in RNase-free water to achieve the concentration in the test diet

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity and stability under bioassay conditions of DvSSJ1_210 dsRNA in the test diet (Treatment 2). Homogeneity assessment on two days of diet preparation is considered representative of all days of diet preparation. The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Bulk dosing solutions were prepared on Day 0 of the CPB bioassay. To generate the test dosing solution for Treatment 2, the test substance was removed from frozen storage, allowed to thaw at ambient temperature, and then diluted in RNase-free water to the appropriate DvSSJ1_210 dsRNA concentration (1.36 ng/ul). The bioassay control dosing solution consisted of RNase-free water. Dosing solutions were prepared and maintained at room temperature when in use; when not in use, dosing solutions were stored refrigerated.

Sample Collection and Characterization of Dosing Solutions and Diets

Sample Collection

Samples of the test dosing solution were collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:13.6 dilution (10 µl test dosing solution to 126 µl QHS) on the day of preparation and following final use. Samples (~300 mg each) of Treatments 1 and 2 were collected during the process of diet distribution for the CPB bioassay, as described **Table 18**. Samples were stored frozen if applicable (-80 °C freezer unit) until analysis.

Table 18: Test Dosing Solution Collection Information for CPB DvSSJ1 dsRNA Bioassay

Treatment	Diet Distribution Day	Number of Samples per Day	Analysis
1	0, 12	5	Absence of DvSSJ1_210 dsRNA
2	0, 12[a]	5 Beginning 5 Middle 5 End	Homogeneity of DvSSJ1_210 dsRNA
	4	15[b]	Stability under bioassay conditions

[a] Homogeneity assessment on two days of diet preparation is considered representative of all days of diet preparation.

[b] Five samples were collected into tubes; the remaining samples were distributed to bioassay plates and placed under bioassay conditions.

Stability under Bioassay Conditions

Samples were collected during one day of diet preparation for verification of stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions. For the Day 0 assessment, five samples were collected into tubes and stored frozen (-80 °C freezer unit) until analysis. For the Days 1-2 assessment, samples (five per day) were randomly distributed to bioassay plates. The bioassay plates were placed under bioassay conditions using the same methods and conditions used in the CPB bioassay, except the plates were not infested with larvae. For each day of stability analysis (Days 1-2), one bioassay plate containing five samples of Treatment 2 was removed from bioassay conditions and stored frozen (- 80 °C freezer unit) until analysis.

Preparation of Sample Homogenates

Diet samples were removed from the freezer, if applicable, in preparation for extraction and analysis (three each per time point from the beginning, middle, and end of diet distribution for homogeneity, three for each Treatment 1 assessment of the absence of DvSSJ1_210 dsRNA, and three for Day 0 bioassay stability). Treatment 2 samples (three for each of Days 1-2) frozen in bioassay plates were removed from bioassay plates and placed into tubes.

A volume of QHS (µl) equal to 10x sample weight (mg) was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were

centrifuged again and the final supernatants (i.e., sample homogeneates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Treatment 1 samples were loaded to the plate undiluted. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the Treatment 2 dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability under bioassay conditions), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solution

The concentration of DvSSJ1_210 dsRNA in test dosing solution was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed for a given diet preparation day.

The acceptable range for each diet preparation day was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since all samples were within the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling day (Days 1 - 2). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

Verification of Absence of DvSSJ1_210 dsRNA in Treatment 1

Absence of DvSSJ1_210 dsRNA in Treatment 1 samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean MFI of lowest Standard Curve Point – 10%)

D3.b. Colorado Potato Beetle DvSSJ1 dsRNA Sensitive Insect Bioassay

The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 used in the CPB bioassay was evaluated by conducting a 14-day bioassay using WCR, a species sensitive to DvSSJ1_210 dsRNA. The WCR bioassay was initiated on Day 0 of the CPB bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)
- Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.05 ng DvSSJ1_210 dsRNA per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Samples were collected from Treatments 1 and 2 on Days 0, 4, 8, and 12 of diet preparation for the CPB bioassay and were used to prepare Treatments A and B for the WCR bioassay as follows:

For each respective treatment, RNase-free water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (*i.e.*, 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the CPB bioassay, resulting in a 5% incorporation of the CPB diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 2 from the CPB bioassay, resulting in a 5% incorporation of the CPB diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small

holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in **Table 13**. Weight data were summarized as means, standard deviations, and ranges in **Table 14**.

D4. Evaluation of the Survival and Weight of Mealworm Fed Artificial Diet Containing DvSSJ1_210 double-stranded RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of Stonefly Heliothis diet.

Test System

The test system was *Tenebrio molitor* (mealworm; Coleoptera: Tenebrionidae; MWM). MWM was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. MWM eggs were obtained from Pioneer Hi-Bred International, Inc. and identity was recorded by study personnel.

Experimental Design

MWM larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained 3 replicates from each treatment. Each treatment was fed to a target of 30 MWM individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour continuous dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.

- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared and characterized under a separate study: "Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA" (See section D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA). A certificate of analysis is included in the study records. The positive control diet (Treatment 3) was also prepared under "Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA" but not characterized.

MWM Bioassay

MWM eggs were incubated in an environmental chamber until the eggs hatched. MWM neonates were used in the bioassay within 48 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of diet was dispensed into wells of the bioassay plates. One MWM neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour continuous dark for a total of 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0. Living MWM larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. A statistical comparison was made between MWM fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

MWM fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of MWM fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatments 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Results and Discussion

The MWM bioassay was run twice. On the initial run, the bioassay did not meet the acceptability criteria as the number of dead and missing larvae in the bioassay control diet (Treatment 1) group exceeded 20%. The bioassay was re-run and the second run met the acceptability criterion for the bioassay control diet; therefore, the results for the second run are included in this report. Data generated for the initial run of the bioassay were retained with the study records.

On the second run of the bioassay, the positive control diet (Treatment 3) group did not meet the acceptability criterion of exceeding 80% mortality. The increased mortality and decreased weight of MWM in Treatment 3 as compared with Treatment 1 (**Table 19** and **Table 20**) indicates MWM were exposed to the boric acid in Treatment 3 by feeding on the provided diet, which demonstrates the experimental design was appropriate.

The mortality of MWM fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 23.3%; P-value = 0.2531) was not significantly greater than those fed the bioassay control diet (Treatment 1; 13.3%), as shown in **Table 19**. The mean weight of MWM fed

Treatment 2 (0.843 mg; P-value = 0.1673) was not significantly less than those fed Treatment 1 (0.923 mg), as shown in **Table 20**. The observed difference in mortality between Treatment 2 and Treatment 1 is not considered biologically relevant in this bioassay, given the mean weight and range of weights.

Conclusion

The results demonstrated the mortality for MWM fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 23.3%) was not significantly greater than the mortality for MWM fed the bioassay control diet (Treatment 1; 13.3%). The mean weight of MWM fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (0.843 mg) was not significantly less than the mean weight of MWM fed Treatment 1 (0.923 mg).

Table 19. Summary Analysis of MWM Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	4	13.3	-
2	Test Diet	1	30	7	23.3	0.2531
3	Positive Control Diet	0[a]	30	18	60.0	-

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

Table 20. Summary Analysis of MWM Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	26	0.923 (0.808 - 1.04)	0.3 - 1.5	-
2	Test Diet	1	23	0.843 (0.721 - 0.966)	0.3 - 1.6	0.1673
3	Positive Control Diet	0[a]	12	0.483 ± 0.119[b]	0.4 - 0.8	-

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.
[b] Standard deviation is provided for mean values not subjected to a *t*-test.

D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA

The objective of this study was to prepare and characterize Stonefly Heliothis diet incorporated with DvSSJ1_210 double-stranded RNA (dsRNA) for use in insect feeding studies.

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. DvSSJ1_210 dsRNA stability was certified under-80 °C freezer unit storage condition.

Carrier

The carrier consisted of Stonefly Heliothis diet

Experimental Design

Bulk DvSSJ1_210 dsRNA test dosing solutions and bioassay control dosing solutions (three each) were prepared and used to make test diets and bioassay control diets. In addition, boric acid dosing solutions were prepared and used to make positive control diets. The target concentration in each positive control diet was determined by the test system used in the respective insect feeding studies. The following treatments were prepared:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3a: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weight)
- Treatment 3b: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weight)
- Treatment 3c: Positive Control Diet (targeting 3750 ng boric acid per mg diet wet weight)
- Treatment 3d: Positive Control Diet (targeting 5000 ng boric acid per mg diet wet weight)
- Treatment 3e: Positive Control Diet (targeting 2500 ng boric acid per mg diet wet weight)
- Treatment 3f: Positive Control Diet (targeting 3125 ng boric acid per mg diet wet weight)

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in each bulk test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in test diets (Treatment 2). The stability under bioassay conditions of DvSSJ1_210 dsRNA in Treatment 2 and the absence of DvSSJ1_210 dsRNA in bioassay control diets (Treatment 1) was also verified. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the

measurement of multiple RNA transcripts/targets simultaneously. Sensitive insect bioassays with WCR were conducted to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

Control of bias during sample collection and analysis was achieved through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. In addition, the use of bulk dosing solutions and bulk standard curve preparation minimized day to day bias. Bias in the WCR bioassays was controlled through the randomization of treatments within blocks.

Preparation of Solutions

Three individual sets of test and bioassay control dosing solutions were prepared. For each test dosing solution preparation, test substance was removed from frozen storage and allowed to thaw at ambient temperature. The test substance was diluted in RNase-free water to create each bulk DvSSJ1_210 dsRNA dosing solution. The DvSSJ1_210 dsRNA concentration in the test dosing solutions (1.33 ng/µl) was targeted to achieve the desired concentration in Treatment 2. The bulk bioassay control dosing solutions used to prepare Treatment 1 consisted of RNase-free water. Dosing solutions were prepared and maintained at room temperature when in use; when not in use, dosing solutions were stored refrigerated.

In addition, positive control dosing solutions containing boric acid were prepared and used to make the positive control diets as described below.

Preparation of Diets

On each day of diet preparation, the test, bioassay control, and the appropriate positive control dosing solutions were each mixed with carrier in a ratio of approximately 3:1 (*i.e.*, 3 ml of dosing solution to 1 g of carrier), generating Treatments 1-3a-f.

Sample Collection

Samples of each test dosing solution preparation were collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:13.3 dilution (10 µl test dosing solution to 123 µl QHS) on the day of preparation and following final use. Samples (~300 mg each) of Treatment 1 and Treatment 2 were collected into tubes during diet distribution as described in Table 21. Samples were stored frozen, if applicable (-80 °C freezer unit), until analysis.

Table 21. Dosing Solutions and Testing Results for WCR Bioassay

Dosing Solution Preparation	Diet Distribution Day	Homogeneity in Treatment 2	Stability under Bioassay Conditions in Treatment 2	Absence in Treatment 1
		Number of samples		
1	0	15[a]	NA	5
	4	NA	NA	NA
	7	NA	NA	NA
	10	15[a]	25[b]	5
2	0	15[a]	NA	5
	4	NA	NA	NA
	7	NA	NA	NA
	11	15[a]	NA	5
3	0	15[a]	NA	5
	4	NA	NA	NA
	7	NA	NA	NA
	11	15[a]	NA	5

Note: NA (not applicable).

[a] Five samples were collected from the beginning of the diet distribution process, five samples from the middle, and five samples from the end. Homogeneity assessment on two days of diet preparation is considered representative of all days of diet preparation.

[b] Five samples were collected into tubes; the remaining samples were distributed to bioassay plates and placed under bioassay conditions.

In addition, a portion each of Treatment 1 and Treatment 2 from each day of diet preparation was collected for use in diets (Treatments A and B, respectively) for sensitive insect bioassays. An individual sensitive insect bioassay using *Diabrotica virgifera virgifera* was conducted for each dosing solution preparation. Preparation of diets for the sensitive insect bioassays is described in section D5.a. WCR DvSSJ1 dsRNA Sensitive Insect Bioassays**.** The positive control diets (Treatments 3a-f) were not characterized.

Stability under Bioassay Conditions

Samples were collected during one day of diet preparation using dosing solution Preparation 1 for verification of stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions. For the Day 0 assessment, five samples were collected into tubes (~300 mg each) and stored frozen (-80 °C freezer unit) until analysis. For the Days 1-4 assessment, samples (~300 mg each) dispensed on the same day as the Day 0 samples were randomly distributed to 12-well bioassay plates (five samples per plate; 20 total) and placed in an environmental growth chamber set to 25 °C, 65% relative humidity, and a 24-hour dark cycle. For each day of stability analysis (Days 1-4), one bioassay plate containing five samples of Treatment 2 was removed from bioassay conditions and stored frozen (- 80 °C freezer unit) until analysis.

Preparation of Sample Homogenates

Diet samples were removed from the freezer in preparation for extraction and analysis (three each from the beginning, middle, and end of diet distribution for homogeneity; three for each Treatment 1 verification of the absence of DvSSJ1; and three for Day 0 bioassay stability). Treatment 2 samples (three for each of Days 1-4) frozen in bioassay plates were removed from bioassay plates and placed into tubes.

QHS (3000 µl) was added to each tube containing diet and samples were vortexed continuously for four minutes and then centrifuged. The supernatants were transferred to individual tubes and centrifuged again. The supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), thawed and warmed, and then diluted as necessary in QHS. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentrations of DvSSJ1_210 dsRNA in the Treatment 2 dosing solutions were interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability under bioassay conditions), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solutions

The concentration of DvSSJ1_210 dsRNA in test dosing solutions was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed for a given diet preparation day.

The acceptable range for each diet preparation day was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using the binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

All homogeneity samples analyzed were within the acceptable range; therefore, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283.

Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling day (Days 1 - 4). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

Demonstration of DvSSJ1_210 dsRNA Activity in WCR Test Diets

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassays. Sensitive insect bioassays were performed with WCR larvae to demonstrate the biological activity of DvSSJ1_210 dsRNA in Treatment 2. Details regarding the sensitive insect bioassays are provided in section D5.a. WCR DvSSJ1 dsRNA Sensitive Insect Bioassays.

Results and Discussion

Three sets of test and bioassay control dosing solutions were prepared and incorporated with Stonefly Heliothis carrier to create artificial diets for use in insect feeding studies.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the three test dosing solutions (from day of preparation and following final use; Table 21) with the following exception: The day of preparation test dosing solution sample for Preparation 3 interpolated at 145% of the expected value. Since this was outside of the acceptance criteria, that dosing solution sample was reanalyzed by dilution by two analysts. Upon further analysis, the sample was still outside of the acceptance criteria. The average of all three analyses was 154% of the expected concentration; however, the sample analyzed following final use for the Preparation 3 test dosing solution was within the acceptance criteria (106% of expected) and homogeneity sample results (MFIs) from both sample collection days from Preparation 3 were comparable. The day of preparation result is attributed to an analytical artifact related only to the sample collected and diluted in QHS on the day of dosing solution preparation, as the sample collected and diluted in QHS following final use met the acceptance criteria. Preparation 3 samples were all analyzed on the same plate, and thus the result that did not meet acceptance criteria was not discovered until all samples had been collected for Preparation 3.

In addition, QuantiGene analysis verified the homogeneity of DvSSJ1_210 dsRNA in test diets (Treatment 2) for each test dosing solution preparation (Table 23).

QuantiGene analysis also verified the absence of DvSSJ1_210 dsRNA in bioassay control diets (Treatment 1) for each bioassay control dosing solution preparation (Table 24). The stability of DvSSJ1_210 dsRNA in Treatment 2 stored under bioassay conditions was also assessed over four days. The test substance was stable in the test diet under bioassay conditions for three days. Diets stored under bioassay conditions did not meet the acceptability criterion on Day 4 (Table 25).

The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 prepared from each test dosing solution preparation was demonstrated by increased mortality and decreased weight of WCR fed test diet containing a portion of Treatment 2 when compared to the bioassay control diet in three individual sensitive insect bioassays (section D5.a. WCR DvSSJ1 dsRNA Sensitive Insect Bioassays). Observed larval mortality and weight data for the WCR sensitive insect bioassays are summarized in Table 26. The WCR bioassays met the acceptability criterion.

Conclusion

Treatments 1, 2, and 3a-3f were prepared and Treatments 1 and 2 were characterized. All treatments are considered suitable for use in insect feeding studies.

Table 22. Verification of DvSSJ1_210 dsRNA Concentration in WCR Test Dosing Solutions

Preparation	Concentration of Test Dosing Solutions used to Prepare Treatment 2[a]					
	Day	% of Expected	Result	Day	% of Expected	Result
1	Day of prep	104	Verified	Final Use	95	Verified
2	Day of prep	126	Verified	Final Use	110	Verified
3	Day of prep	154[b]	> 130% of expected	Final Use	106	Verified

[a] Concentrations of DvSSJ1_210 dsRNA in test dosing solutions within 70-130% of the expected value were considered verified.
[b] Average of three results.

Table 23. Verification of Homogeneity of DvSSJ1_210 dsRNA in WCR Test Diets

Preparation	Homogeneity[a] of Treatment 2			
	Verification	Result	Verification	Result
1	1st	Verified	2nd	Verified
2	1st	Verified	2nd	Verified
3	1st	Verified	2nd	Verified

[a] Homogeneity was considered verified for Treatment 2 if the value of each sample was within 70-130% of the mean value of all samples analyzed for that diet preparation.

Table 24. Verification of Absence of DvSSJ1_210 dsRNA in WCR Bioassay Control Diets

Preparation	Absence of DvSSJ1_210 dsRNA in Treatment 1			
	Verification	Result	Verification	Result
1	1st	Absent	2nd	Absent
2	1st	Absent	2nd	Absent
3	1st	Absent	2nd	Absent

Table 25. Verification of DvSSJ1_210 dsRNA Stability under WCR Bioassay Conditions in Test Diet

Dosing Solution Preparation	Bioassay Stability in Treatment 2	
	Bioassay Stability Day	Average % of Day 0 Mean[a]
1	Day 0	NA
	Day 1	75
	Day 2	74
	Day 3	72
	Day 4	63

Note: Not applicable (NA).
[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

Table 26. Summary of WCR Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1_210 dsRNA/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
Bioassay 1							
A	Bioassay Control Diet	0	29[a]	13.8	25	1.88 ± 0.681	0.6 - 3.2
B	Test Diet	0.1	30	96.7	1	0.200	NA
Bioassay 2							
A	Bioassay Control Diet	0	30	13.3	26	1.90 ± 0.641	1.0 - 3.0
B	Test Diet	0.1	29[a]	79.3	6	0.383 ± 0.0983	0.3 - 0.5
Bioassay 3							
A	Bioassay Control Diet	0	30	23.3	23	1.73 ± 0.756	0.5 - 3.1
B	Test Diet	0.1	30	66.7	10	0.410 ± 0.173	0.2 - 0.6

Note: The sensitive insect bioassay number (1, 2, and 3) corresponds to the dosing solution preparation used to prepare Treatments 1 and 2 incorporated in the sensitive insect bioassay diets. Treatments A and B used in each sensitive insect bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet. Not applicable (NA); there was only one surviving organism in the test diet group.
[a] Organisms counted as missing during a bioassay were not included in the total number of observations for a given treatment.

D5.a. WCR DvSSJ1 dsRNA Sensitive Insect Bioassays

The biological activity of DvSSJ1_210 dsRNA in Treatment 2 prepared from three individual preparations of test dosing solutions was evaluated by conducting three individual 14-day bioassays using WCR, a species sensitive to DvSSJ1_210 dsRNA. Each WCR bioassay was initiated on the first day of diet preparation (considered Day 0) and refed every three to four days.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel. The carrier for the WCR bioassays consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

For each WCR bioassay, larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)
- Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.1 ng DvSSJ1_210 dsRNA per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals for each bioassay.

Diet Preparation

Samples were collected from Treatments 1 and 2 on each day of diet preparation and were used to prepare Treatments A and B for the three individual WCR bioassays as follows:

For each respective treatment, RNase-free water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (*i.e.*, 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1, resulting in a 10% incorporation of Treatment 1 by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 2, resulting in a 10% incorporation of Treatment 2 by wet weight of the WCR diet.

WCR Bioassays

For each bioassay, WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in each bioassay within 24 hours of hatching. On Day 0 for each bioassay, approximately 300 µl (*i.e.*, 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. Each bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 14 days. Every three to four days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates,

missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, each bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated a bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for each sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in **Table 26**. Weight data were summarized as means, standard deviations, and ranges and are presented in **Table 26**.

D6. Evaluation of the Survival and Weight of Superworm Fed Artificial Diet Containing DvSSJ1 210 bp ds RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of Stonefly Heliothis diet.

Test System

The test system was *Zophobas morio* (superworm; Coleoptera: Tenebrionidae; SWM). SWM was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. SWM eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

SWM larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained 3 replicates from each treatment. Each treatment was fed to a target of 30 SWM individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared and characterized under a separate study: "Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA" (See **D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA**). A certificate of analysis is included in the study records. The positive control diet (Treatment 3) was also prepared under were prepared and characterized under a separate study: "Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA" (See **D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA**) but not characterized.

SWM Bioassay

SWM eggs were incubated in an environmental chamber until the eggs hatched. SWM neonates were used in the bioassay within 36 hours of hatching.

On Day 0, approximately 300 µl (*i.e.*, 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One SWM neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0. Living SWM larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. A statistical comparison was made between SWM fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of *SWM* fed the artificial diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of SWM fed the artificial diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatment 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Results and Discussion

The SWM bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in **Table 27**.

The mortality of SWM fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 6.90%; P-value = 0.6811) was not significantly greater than those fed the bioassay control diet (Treatment 1; 6.67%)

The mean weight of SWM fed Treatment 2 (1.21 mg; P-value = 0.5547) was not significantly less than those fed Treatment 1 (1.20 mg) (**Table 28**)

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of SWM.

Table 27. Summary Analysis of SWM Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	2	6.67	--
2	Test Diet	1	29[a]	2	6.90	0.6811
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.
[b] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

Table 28. Summary Analysis of SWM Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	1.20 (1.07 - 1.33)	0.8 - 2.0	--
2	Test Diet	1	27	1.21 (1.04 - 1.39)	0.5 - 2.3	0.5547
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving *SWM* in Treatment 3.
[a] Treatment 3 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

D7. Evaluation of the Survival and Weight of Red Flour Beetle Fed an Artificial Diet Containing DvSSJ1 210bp ds RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of red flour beetle diet prepared by Pioneer Hi-Bred International, Inc.

Dosing Solutions

The bioassay control dosing solutions used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solutions used to prepare Treatment 2 consisted of the test substance diluted in RNase-free water to achieve the concentration in the test diet.

The positive control dosing solutions used to prepare Treatment 3 consisted of boric acid (H_3BO_3) and RNase-free water.

Test System

The test system was *Tribolium casteneum* (red flour beetle; Coleoptera: Tenebrionidae; RFB). was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. RFB eggs for this study were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

RFB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 3000 ng boric acid per mg diet dry weight)

Three independent bioassays were conducted. For each bioassay, treatments were arranged in a generalized randomized block design with a total of 3 blocks. Each block consisted of a tray containing 1-oz plastic cups and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 RFB individuals. The bioassays were conducted in an environmental chamber set at 30 °C, 70% relative humidity, and continuous dark. Larvae were refed every 3 to

4 days. After 14 days, each bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated a bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in each test dosing solution, the homogeneity of DvSSJ1_210 dsRNA in each test diet (Treatment 2), and the stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions and frozen storage. The absence of DvSSJ1_210 dsRNA in each bioassay control diet (Treatment 1) was also verified. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

Bias in the RFB bioassays and sensitive insect bioassays was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria.

Diet Generation

Each diet was prepared in bulk for the duration of each bioassay. Dosing solutions for Treatments 1 and 2 for each bioassay were prepared as described in section D7.a. Red Flour Beetle DvSSJ1 dsRNA Bioassay Analytical Phase. The positive control dosing solution for Treatment 3 for each bioassay was prepared by solubilizing and diluting boric acid in RNase-free water to achieve a nominal concentration of 3000 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 2:1 ratio (*i.e.*, 2 ml dosing solution to 1 g carrier) to generate Treatments 1-3. After mixing, diets were lyophilized, aliquoted into individual storage tubes, and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting for each bioassay, samples of Treatments 1 and 2 were collected for characterization of diets as described in section D7.a. Red Flour Beetle DvSSJ1 dsRNA Bioassay Analytical Phase. In addition, a portion each of Treatment 1 and Treatment 2 was collected for use in diets for the sensitive insect bioassays and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassays is described in section D7.a. Red Flour Beetle DvSSJ1 dsRNA Bioassay Analytical Phase. The positive control diet (Treatment 3) was not characterized.

RFB Bioassay

Three bioassays were conducted to determine the response of RFB to DvSSJ1_210 dsRNA exposure via oral ingestion. Individual batches of RFB eggs were used in each bioassay. RFB eggs were incubated in an environmental chamber until the eggs hatched and neonates were used in a bioassay within 24 hours of hatching.

On Day 0 for each bioassay, diet aliquots were removed from frozen storage and distributed into 30 individual plastic cups. One RFB neonate was placed in each cup containing diet, and then the cup was sealed with a lid. The bioassays were conducted in an environmental chamber set at 30 °C, 70% relative humidity, and continuous dark for a total of 14 days. Every 3 to 4 days, new bioassay cups were prepared as described for Day 0. Living RFB larvae were transferred to the new cups, missing or dead larvae were recorded, and the freshly prepared cups were placed in the environmental chamber. After 14 days, each bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only cups that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a cup or lost in transfer, or cups containing more than one organism, were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparison was made between RFB fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

RFB fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of Treatment groups 1 and 2; therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition. That is, to test if the weight of RFB larvae fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) was less than the weight of larvae fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatment 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Demonstration of DvSSJ1_210 dsRNA Activity in the *RFB* Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassays using *Diabrotica virgifera virgifera* (WCR) larvae to demonstrate the biological activity of the DvSSJ1_210 dsRNA used in Treatment 2 of the RFB bioassays. Details regarding the sensitive insect bioassays are provided in section D7.b. Red Flour Beetle DvSSJ1 dsRNA Sensitive Insect Bioassay.

Results and Discussion

The initial RFB bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 30. However, two additional RFB bioassays were conducted to further characterize the biological variability of RFB. Those bioassays also met the acceptability criteria and data from all three runs are included in this report.

The mortality of RFB fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet in the three bioassays (Treatment 2; 14.8%, 0% and 3.33%; P-value = 0.0515, 1.0000, and 0.7542, respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1; 0%, 0%, and 3.33%), as shown in Table 30.

The mean weight of RFB fed Treatment 2 (1.90, 2.48, and 2.47 mg; P-value = 0.2738, 0.5973, and 0.6877, respectively) was not significantly less than the mean weight of those fed Treatment 1 (2.00, 2.45, and 2.41 mg), as shown in Table 31.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in each Treatment 2 test dosing solution (**Table 33**). The homogeneity of DvSSJ1_210 dsRNA in Treatment 2 and absence of DvSSJ1_210 dsRNA in Treatment 1 was also verified for each bioassay (**Table 33**). The stability of DvSSJ1_210 dsRNA in Treatment 2 stored under bioassay conditions was verified over four days and frozen storage stability was verified over 41 days (**Table 34**).

Observed larval mortality and weight data for the WCR sensitive insect bioassays are summarized in **Table 32**. The WCR bioassays met the acceptability criterion. The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 of the RFB bioassays was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B) compared to those fed the bioassay control diet (Treatment A).

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of RFB.

D7.a. Red Flour Beetle DvSSJ1 dsRNA Bioassay Analytical Phase

The following dosing solutions were prepared for the RFB bioassays:

Bioassay control dosing solutions used to prepare Treatment 1 consisting of RNase-free water

Test dosing solutions used to prepare Treatment 2 consisting of test substance diluted in RNase-free water to achieve the concentration in the test diet

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in each test dosing solution, the homogeneity of DvSSJ1_210 dsRNA in each test diet (Treatment 2), and the stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions and frozen storage. The absence of DvSSJ1_210 dsRNA in each bioassay control diet (Treatment 1) was also verified. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation for each RFB bioassay. To generate the test dosing solutions for Treatment 2, the test substance was removed from frozen storage, allowed to thaw at ambient temperature, and then diluted in RNase-free water to the appropriate DvSSJ1_210 dsRNA concentration (0.5 ng/µl). The bioassay control dosing solutions consisted of RNase-free water. Dosing solutions were prepared at room temperature.

Sample Collection and Characterization of Dosing Solutions and Diets

Sample Collection

A sample of each test dosing solution was collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:5 dilution factor (30 µl test dosing solution to 120 µl QHS) on the day of preparation. Samples (~50 mg each) of Treatments 1 and 2 were collected into pre-weighed tubes during the process of diet aliquoting for the RFB bioassay, as described in **Table 29**. The tubes containing diet were weighed again in order to obtain the exact sample weight. Samples were stored frozen if applicable (-80 °C freezer unit) until analysis.

Table 29. RFB Bioassay Diet Homogeniety and Stability Results

Bioassay	Homogeneity in Treatment 2	Stability under Bioassay Conditions in Treatment 2	Frozen Storage Stability in Treatment 2	Absence in Treatment 1
	Number of Samples			
1	15[a]	25[b]	33[c]	5
2	15[a]	NA	NA	5
3	15[a]	NA	NA	5

Note: NA (not applicable). Frozen storage refers to storage in a -80 °C freezer unit.

[a] Five samples were collected from the beginning of the diet aliquoting process, five samples from the middle, and five samples from the end.

[b] Five samples were collected into tubes; the remaining 20 samples were distributed to plastic cups.

[c] Three samples were extracted on the day of diet aliquoting for Day 0 freezer stability analysis. Thirty samples were collected and placed in the freezer (-80 °C freezer unit). Approximately weekly for six weeks, three samples were removed from the freezer and extracted.

Stability under Bioassay Conditions

Samples were collected for verification of stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions. For the Day 0 assessment, five samples were collected into pre-weighed tubes and stored frozen (-80 °C freezer unit) until analysis. For the Days 1-4 assessment, samples (five per day) were randomly distributed to plastic cups and placed under bioassay conditions using the same methods and conditions used in the RFB bioassay, except the cups were not infested with larvae, the diet amount was increased, and diet was placed in a cap within each cup for ease of recovery for analytical purposes. For each day of stability analysis, five cups containing Treatment 2 were removed from bioassay conditions. The diet in each cup was transferred to an individual pre-weighed tube, and the tube containing diet sample reweighed before being stored frozen (-80 °C freezer unit) until analysis.

Preparation of Sample Homogenates

Diet samples were removed from the freezer, as applicable, in preparation for extraction and analysis (three each from the beginning, middle, and end of diet aliquoting for each homogeneity verification, three for each Treatment 1 verification of the absence of DvSSJ1_210 dsRNA, and three for each stability time point and condition).

A volume of QHS (µl) equal to 10x sample weight (mg) was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were centrifuged again and the final supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Treatment 1 samples were loaded to the plate undiluted. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present

in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in each Treatment 2 dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solutions

The concentration of DvSSJ1_210 dsRNA in test dosing solution was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed for a given bioassay.

The acceptable range was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Verification of Absence of DvSSJ1_210 dsRNA in Treatment 1

Absence of DvSSJ1_210 dsRNA in Treatment 1 samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = Mean MFI of lowest Standard Curve Point – 10%

Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling day (Days 1 - 4). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

Verification of DvSSJ1_210 dsRNA Stability under Frozen Storage Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by time point (Days 7, 13, 19, 27, 34, and 41). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

For the Day 41 timepoint, two of three samples were used for stability calculation. The remaining sample was excluded because the result (183% of Day 0 mean) was 63% different relative to the average of the other two samples.

D7.b. Red Flour Beetle DvSSJ1 dsRNA Sensitive Insect Bioassay

The biological activity of the DvSSJ1_210 dsRNA in each Treatment 2 used in the RFB bioassays was evaluated by conducting an individual 14-day bioassay using WCR, a species sensitive to DvSSJ1_210 dsRNA. Each WCR bioassay was initiated after completion of the corresponding RFB bioassay.

Western corn rootworm (WCR; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)

Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.0855 ng DvSSJ1_210 dsRNA per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 2 during diet aliquoting for each RFB bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B for a corresponding WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the RFB bioassay, resulting in a 30% incorporation of the RFB diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 2 from the RFB bioassay, resulting in a 30% incorporation of the RFB diet by dry weight of the WCR diet.

For each respective treatment, RNase-free water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassays

For each individual bioassay, WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassays within 24 hours of hatching. On Day 0 of each bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. Each bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, each bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well or lost in transfer, or wells that contained more than one organism, were excluded from statistical analysis.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassays were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of each bioassay multiplied by 100 and are presented in **Table 32**. Weight data were summarized as means, standard deviations, and ranges in **Table 32**.

Table 30. Summary Analysis of RFB Mortality Results

Bioassay	Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	1	Bioassay Control Diet	0	28[a]	0	0	--
	2	Test Diet	1	27[a]	4	14.8	0.0515
	3	Positive Control Diet	0[b]	28[a]	28	100	--
2	1	Bioassay Control Diet	0	30	0	0	--
	2	Test Diet	1	29[a]	0	0	1.0000
	3	Positive Control Diet	0[b]	29[a]	29	100	--
3	1	Bioassay Control Diet	0	30	1	3.33	--
	2	Test Diet	1	30	1	3.33	0.7542
	3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay or lost in transfer, or cups containing more than one organism, were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 3000 ng boric acid per mg diet dry weight.

Table 31. Summary Analysis of RFB Weight Results

Bioassay	Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	1	Bioassay Control Diet	0	28	2.00 (1.82 - 2.18)	1.0 - 2.7	--
	2	Test Diet	1	23	1.90 (1.61 - 2.19)	0.3 - 3.0	0.2738
	3	Positive Control Diet	0[a]	0	NA	NA	--
2	1	Bioassay Control Diet	0	30	2.45 (2.32 - 2.59)	1.5 - 3.1	--
	2	Test Diet	1	29	2.48 (2.35 - 2.61)	1.9 - 3.7	0.5973
	3	Positive Control Diet	0[a]	0	NA	NA	--
3	1	Bioassay Control Diet	0	29	2.41 (2.19 - 2.62)	0.1 - 3.3	--
	2	Test Diet	1	29	2.47 (2.30 - 2.64)	1.7 - 3.4	0.6877
	3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving *Tribolium casteneum* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 3000 ng boric acid per mg diet dry weight.

Table 32. Summary of Sensitive Insect Bioassay Results (for RFB Bioassay)

Bioassay	Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
							Mean ± Standard Deviation	Range
1	A	Bioassay Control Diet	0	30	30.0	21	1.26 ± 0.851	0.1 - 3.0
	B	Test Diet	0.0855	30	76.7	7	0.400 ± 0.163	0.2 - 0.7
2	A	Bioassay Control Diet	0	27[a]	3.70	26	1.60 ± 0.677	0.4 - 2.8
	B	Test Diet	0.0855	30	76.7	7	0.771 ± 0.407	0.2 - 1.4
3	A	Bioassay Control Diet	0	30	16.7	25	1.79 ± 0.606	0.7 - 2.8
	B	Test Diet	0.0855	29[a]	58.6	12	0.383 ± 0.119	0.2 - 0.6

Note: Treatments A and B used in each sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the respective *Tribolium casteneum* bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

[a] Organisms counted as missing during the bioassay or lost in transfer, or wells that contained more than one organism, were not included in the total number of observations for a given treatment.

Table 33. Verification of DvSSJ1_210 dsRNA Concentration in Test Dosing Solutions, Homogeneity in Test Diets, and Absence in Bioassay Control Diets for RFB

Analysis	Bioassay	Result
Test Dosing Solution Concentration Verification	1	105% of expected[a]
	2	105% of expected[a]
	3	106% of expected[a]
Homogeneity in Test Diet (Treatment 2)	1	Verified[b]
	2	Verified[b]
	3	Verified[b]
Absence in Bioassay Control Diet (Treatment 1)	1	Verified[c]
	2	Verified[c]
	3	Verified[c]

[a] Concentrations within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified for a given Treatment 2 diet preparation if the value of each sample was within 70-130% of the mean value of all samples analyzed for that diet preparation.

[c] Absence of DvSSJ1_210 dsRNA in Treatment 1 was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

Table 34. Verification of DvSSJ1_210 dsRNA Stability in RFB Test Diet (Treatment 2)

Stability under Bioassay Conditions		Frozen Storage Stability	
Bioassay Stability Day	Average % of Day 0 Mean[a]	Storage Day	Average % of Day 0 Mean[a]
Day 0	NA	0	NA
Day 1	96	7	105
Day 2	94	13	101
Day 3	96	19	103
Day 4	103	27	108
--	--	34	114
		41	112[b]

Note: Frozen storage stability samples were stored in a -80 °C freezer unit. Not applicable (NA).

[a] Diet samples were considered stable if the average percentage for each sampling time point and condition was ≥ 70% of the Day 0 mean.

[b] Value is the average of two samples. The percentage of Day 0 mean for all other sampling time points and condition is the average of three samples.

D8. Evaluation of Survival and Weight of Mexican Bean Beetle Fed an Artificial Diet Containing DvSSJ1 210 bp ds RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted primarily of Stonefly Heliothis diet.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solution used to prepare Treatment 2 consisted of the test substance diluted in RNase-free water to achieve the concentration in the test diet.

The positive control dosing solution used to prepare Treatment 3 consisted of boric acid (H_3BO_3) and RNase-free water.

Test System

The test system was *Epilachna varivestis* (Mexican bean beetle; Coleoptera: Coccinellidae; MBB) was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. MBB eggs were obtained from New Jersey Department of Agriculture (Trenton, NJ, USA) and identity was recorded by study personnel.

Experimental Design

MBB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 1250 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained 3 replicates from each treatment. Each treatment was fed to a target of 30 MBB individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous

dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in the test diet (Treatment 2). In addition, the stability under bioassay conditions of DvSSJ1_210 dsRNA in Treatment 2 was assessed. The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

Bias in the MBB bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. In addition, the use of bulk dosing solutions and bulk standard curve preparation minimized day to day bias.

Diet Generation

Bulk dosing solutions for Treatments 1 and 2 were prepared and maintained as described in Appendix A. The boric acid dosing solution for Treatment 3 was prepared on each day of diet preparation by diluting boric acid in RNase-free water to achieve a nominal concentration of 1250 ng/mg diet wet weight. On each day of diet preparation, each dosing solution was mixed with carrier in a 3:1 ratio (*i.e.*, 3 ml of dosing solution to 1 g of carrier), generating Treatments 1-3.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1 and 2 were collected for characterization of diets as described in section D8.a. Mexican Bean Beetle DvSSJ1 dsRNA Bioassay Analytical Phase. In addition, a portion of Treatment 1 and Treatment 2 from each day of diet preparation was collected for use in diets for the sensitive insect bioassay as described in section D8.b. Mexican BeanBeetle DvSSJ1 dsRNA Sensitive Insect Bioassay. The positive control diet (Treatment 3) was not characterized.

MBB Bioassay

MBB eggs were incubated in an environmental chamber until the eggs hatched. MBB neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One MBB neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark for a total of 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living MBB larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. A statistical comparison was made between MBB fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

MBB fed the artificial diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

Significance was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of MBB fed the artificial diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatments 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Demonstration of DvSSJ1_210 dsRNA Activity in the MBB Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay. A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the DvSSJ1_210 dsRNA used in Treatment 2 in the MBB bioassay.

Results and Discussion

The MBB bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 35.

The mortality of MBB fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 0%; P-value = 1.0000) was not significantly greater than those fed the bioassay control diet (Treatment 1; 0%), as shown in Table 35.

The mean weight of MBB fed Treatment 2 (3.11 mg; P-value = 0.2693) was not significantly less than the mean weight of those fed Treatment 1 (3.22 mg), as shown in Table 36.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in Treatment 2 (Table 38). Homogeneity assessment on one day of diet preparation is considered representative of all days of diet preparation. The absence of DvSSJ1_210 dsRNA in Treatment 1 was also verified (Table 38).

In addition, the stability of DvSSJ1_210 dsRNA in Treatment 2 stored under bioassay conditions was assessed over four days. The test substance was stable in the test diet under bioassay conditions for one day. Diets stored under bioassay conditions did not meet the acceptability criteria on Days 2-4 (Table 39); however, organisms were refed with fresh diets every three to four days and biological activity was demonstrated during the sensitive insect bioassay.

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 35 and Table 36. The WCR bioassay met the acceptability criterion. The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 of the MBB bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in Table 37 and section D8.b. Mexican BeanBeetle DvSSJ1 dsRNA Sensitive Insect Bioassay) when compared to the bioassay control diet.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of MBB.

Table 35. Summary Analysis of MBB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	29[a]	0	0	--
2	Test Diet	1	30	0	0	1.0000
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 1250 ng boric acid per mg diet wet weight.

Table 36. Summary Analysis of MBB DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	29	3.22 (2.90 - 3.55)	1.7 - 5.2	--
2	Test Diet	1	30	3.11 (2.89 - 3.33)	2.3 - 5.0	0.2693
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving MBB in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 1250 ng boric acid per mg diet wet weight.

Table 37. Summary of MBB DvSSJ1 dsRNA Bioassay Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	29[a]	10.3	26	1.67 ± 0.835	0.2 - 2.7
B	Test Diet	0.1	29[a]	96.6	1	0.400[b]	0.4

Note: Treatments A and B used in the sensitive insect (WCR) bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the MBB bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

[a] Organisms counted as missing during the bioassay, or wells that contained more than one organism, were not included in the total number of observations for a given treatment.

[b] The reported mean is the weight value of the one surviving larva after the 14-day feeding period; no standard deviation was calculated

Table 38. Verification of DvSSJ1_210 dsRNA Concentration in Test Dosing Solution, Homogeneity in Test Diet, and Absence in Bioassay Control Diet for MBB Bioassay.

Analysis	Diet Preparation Day	Diet Sample	Result
Test Dosing Solution Concentration Verification	0	NA	Verified[a]
	After final use		Verified[a]
Homogeneity in Test Diet (Treatment 2)	0	Beginning	Verified[b]
		Middle	
		End	
	11	Beginning	Verified[b]
		Middle	
		End	
Absence in Bioassay Control Diet (Treatment 1)	0	NA	Verified
	11		Verified

Note: Not applicable (NA). Beginning, middle, and end refer to the stages in the diet aliquoting process at which homogeneity samples were collected.

[a] The test dosing solution concentration was 116% and 115% of the expected DvSSJ1_210 dsRNA concentration for the Day 0 and after final use dosing solutions, respectively. Concentrations within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified for a given Treatment 2 diet preparation if the value of each sample was within 70-130% of the mean value of all samples analyzed for that diet preparation

Table 39. Assessment of DvSSJ1_210 dsRNA Stability under MBB Bioassay Conditions in Test Diet

Bioassay Stability Day	Average % of Day 0 Mean[a]
Day 0	NA
Day 1	76
Day 2	64
Day 3	55
Day 4	49

Note: NA (not applicable).

[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean. Organisms were refed with fresh diets every three to four days.

D8.a. Mexican Bean Beetle DvSSJ1 dsRNA Bioassay Analytical Phase

The following dosing solutions were prepared for the MBB bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water

Test dosing solution used to prepare Treatment 2 consisting of test substance diluted in RNase-free water to achieve the concentration in the test diet

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in the test diet (Treatment 2). Homogeneity assessment on one day of diet preparation is considered representative of all days of diet preparation. In addition, the stability under bioassay conditions of DvSSJ1_210 dsRNA in Treatment 2 was assessed. The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Bulk dosing solutions were prepared on Day 0 of the MBB bioassay. To generate the test dosing solution for Treatment 2, the test substance was removed from frozen storage, allowed to thaw at ambient temperature, and then diluted in RNase-free water to the appropriate DvSSJ1_210 dsRNA concentration (1.33 ng/ul). The bioassay control dosing solution consisted of RNase-free water. Dosing solutions were prepared and maintained at room temperature when in use; when not in use, dosing solutions were stored refrigerated.

Sample Collection

Samples of the test dosing solution were collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:13.3 dilution factor (10 µl test dosing solution to 123 µl QHS) on the day of preparation and following final use. Samples (~300 mg each) of Treatments 1 and 2 were collected into tubes during the process of diet distribution for the MBB bioassay, as described in Table 40. Samples were stored frozen, if applicable (-80 °C freezer unit), until analysis.

Table 40. Sampling of MBB Test Dosing Solutions

Treatment	Diet Distribution Day	Number of Samples per Day	Analysis
1	0, 11	5	Verify absence of DvSSJ1_210 dsRNA
2	0, 11[a]	5 Beginning	Homogeneity of DvSSJ1_210 dsRNA
		5 Middle	
		5 End	
	7	25[b]	Stability under bioassay conditions

[a] Homogeneity assessment on two days of diet preparation is considered representative of all days of diet preparation.

[b] Five samples were collected into tubes; the remaining samples were distributed to bioassay plates and placed under bioassay conditions.

Stability under Bioassay Conditions

Samples were collected during one day of diet preparation for verification of stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions. For the Day 0 assessment, five samples were collected into tubes and stored frozen (-80 °C freezer unit) until analysis. For Days 1-4 assessment, samples (five per day) were randomly distributed to bioassay plates and placed under bioassay conditions using the same methods and conditions used in the MBB bioassay, except they were not infested with larvae. For each day of stability analysis (Days 1-4), one bioassay plate containing five samples of Treatment 2 was removed from bioassay conditions and stored frozen (- 80 °C freezer unit) until analysis.

Preparation of Sample Homogenates

Diet samples were removed from the freezer in preparation for extraction and analysis (three each from the beginning, middle, and end of diet distribution for homogeneity, three for each Treatment 1 verification of the absence of DvSSJ1_210 dsRNA, and three for Day 0 bioassay stability). Treatment 2 samples (three for each of Days 1-4) frozen in bioassay plates were removed from bioassay plates and placed into tubes.

QHS (3000 µl) was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were transferred to individual tubes and centrifuged again. The supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Treatment 1 samples were loaded to the plate undiluted. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the Treatment 2 dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability under bioassay conditions), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solution

The concentration of DvSSJ1_210 dsRNA in the test dosing solution was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed for a given diet preparation day.

The acceptable range for each diet preparation day was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples for each homogeneity verification was determined to be sufficient to conclude homogeneity.

Assessment of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling day (Days 1 - 4). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

D8.b. Mexican BeanBeetle DvSSJ1 dsRNA Sensitive Insect Bioassay

The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 used in the MBB bioassay was evaluated by conducting a 14-day bioassay using WCR, a species sensitive to DvSSJ1_210 dsRNA. The WCR bioassay was initiated on Day 0 of the MBB bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the *WCR* bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)
- Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.1 ng DvSSJ1_210 dsRNA per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Samples were collected from Treatments 1 and 2 on each day of diet preparation for the MBB bioassay and were used to prepare Treatments A and B for each day of diet preparation for the WCR bioassay as follows:

For each respective treatment, RNase-free water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the MBB bioassay, resulting in a 10% incorporation of the MBB diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 2 from the MBB bioassay, resulting in a 10% incorporation of the MBB diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells

of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100 and are presented in Table 37 Weight data were summarized as means, standard deviations, and ranges and are also presented in Table 37.

D9. Evaluation of the Survival, Weight, and Development of *Hippodamia convergens* Fed an Artificial Diet Containing DvSSJ1_210 double-stranded RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of *Ephestia* (*Ephestia kuehniella* sp.) eggs.

Test System

The test system was CNV (convergent lady beetle; Coleoptera: Coccinellidae; CNV). CNV was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. CNV eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

CNV larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 15,000 ng boric acid per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a stack of six Petri dishes secured together and contained two replicates from each treatment. Each treatment was fed to a target of 30 CNV individuals. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 17, all organisms had either died or emerged and the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared and characterized under a separate study "Preparation and Characterization of an Artificial Diet Containing *Ephestia* Eggs Incorporated with DvSSJ1 210bp DvSSJ1 dsRNA" (See section D10. Preparation and Characterization of an Artificial Diet Containing *Ephestia* Eggs Incorporated with DvSSJ1 210bp ds RNA). The positive control diet (Treatment 3) was also prepared under "Preparation and Characterization of an Artificial Diet Containing *Ephestia* Eggs Incorporated with DvSSJ1 210bp DvSSJ1 dsRNA" but was not characterized.

CNV Bioassay

CNV eggs were incubated in an environmental chamber until the eggs hatched. CNV neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet aliquots were removed from frozen storage and approximately 120 mg were aliquoted into individual small caps. A cap containing the appropriate diet treatment and a moisture source (a tube filled with 0.5% agar) were distributed to each Petri dish utilized in the bioassay. One CNV neonate was placed in each dish and the dishes were stacked into blocks and secured together. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Every 3-4 days, bioassay dishes were removed from the environmental chamber, pupation was assessed, and missing or dead organisms were recorded. For each living organism that had not yet pupated or died, old diet was removed from the dish and a new diet-filled cap was prepared and distributed as described for Day 0, prior to returning the dishes to the environmental chamber. Once pupation was observed, organisms were assessed daily for adult emergence. CNV adults were weighed within approximately 24 hours of emergence. On Day 17, all larvae had either died or emerged as adults; therefore, the bioassay was complete and mortality was assessed. Only dishes that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a dish were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparison was made between CNV fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

CNV fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused growth inhibition; *i.e.*, lower weight of CNV fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatment 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Number of Days to Adult Emergence

The normality assumption necessary for a two-sample *t*-test was not satisfied by data distributions of treatment groups 1 and 2; therefore, a non-parametric two-sample test was conducted to examine if exposure to DvSSJ1_210 dsRNA caused developmental delay.

The corresponding hypothesis test was

$H_0 : F_T(x) = F_c(x)$ *vs.* $H_a : F_T(x) > F_c(x)$

Where $F_T(x)$ is the cumulative distribution of the number of days to adult emergence of *CNV* fed the artificial insect diet containing DvSSJ1_210 dsRNA, and $F_c(x)$is the cumulative distribution of the number of days to adult emergence of individuals fed the bioassay control diet. Significance would indicate that insects fed the diet containing DvSSJ1_210 dsRNA would have a greater probability to take longer to emerge than those fed the bioassay control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Results and Discussion

The CNV bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 41.

The mortality of CNV fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 7.41%; P-value = 0.6531) was not significantly greater than those fed the bioassay control diet (Treatment 1; 6.67%), as shown in Table 41.

The mean weight of CNV fed Treatment 2 (18.0 mg; P-value = 0.1157) was not significantly less than those fed Treatment 1 (19.1 mg), as shown in Table 42.

CNV fed Treatment 2 (median 15 days; Wilcoxon test P-value = 0.3972) and Treatment 1 (median 15 days) did not significantly differ in the probability to take longer to emerge, as shown in Table 43. In addition, the Siegel-Tukey test (P-value = 0.6016) did not show significant evidence that the scales of the two populations differed.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival, weight, or adult emergence of CNV.

Table 41. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	2	6.67	--
2	Test Diet	1	27[a]	2	7.41	0.6531
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 42. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	19.1 (17.8 - 20.3)	14.3 - 26.2	--
2	Test Diet	1	25	18.0 (16.7 - 19.3)	12.8 - 24.5	0.1157
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 43. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Results for Days to Adult Emergence

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Data Points	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel Tukey Test P-Value
1	Bioassay Control Diet	0	28	15.1 ± 0.786	15	13 - 17	--	--
2	Test Diet	1	25	15.2 ± 0.879	15	14 - 17	0.3972	0.6016
3	Positive Control Diet	0[a]	0	NA	NA	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

D10. Preparation and Characterization of an Artificial Diet Containing *Ephestia* Eggs Incorporated with DvSSJ1 210bp ds RNA

The objective of this study was to prepare and characterize *Ephestia* egg diet incorporated with DvSSJ1_210 double-stranded RNA (dsRNA) for use in insect feeding studies.

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of *Ephestia* (*Ephestia kuehniella* sp.) eggs.

Experimental Design

DvSSJ1_210 dsRNA test dosing solutions and bioassay control dosing solutions (two each) were prepared and used to make test diets and bioassay control diets. In addition, boric acid dosing solutions were prepared and used to make positive control diets. The target concentration in each positive control diet was determined by the test system used in the respective insect feeding studies. The following treatments were prepared:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3a: Positive Control Diet (targeting 15,000 ng boric acid per mg diet dry weight)
- Treatment 3b: Positive Control Diet (targeting 20,000 ng boric acid per mg diet dry weight)

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in each test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in each test diet (Treatment 2). The stability under bioassay conditions and frozen storage stability of DvSSJ1_210 dsRNA in Treatment 2 and the absence of DvSSJ1_210 dsRNA in each bioassay control diet (Treatment 1) was also verified. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay that combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously. Sensitive insect bioassays with WCR (D10.a. Western Corn Rootworm DvSSJ1 dsRNA Sensitive Insect Bioassays) were used to demonstrate the biological activity of DvSSJ1_210 dsRNA in Treatment 2.

Control of bias during sample collection and analysis was achieved through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. In addition, bulk standard curve preparation minimized day to day bias. Bias in the *WCR* bioassays was controlled through the randomization of treatments within blocks.

Preparation of Solutions

Two individual sets of test and bioassay control dosing solutions were prepared. For each test dosing solution preparation, test substance was removed from frozen storage. The test substance was diluted in RNase-free water to create each DvSSJ1_210 dsRNA dosing solution. The DvSSJ1_210 dsRNA concentration in the test dosing solutions (1.33 ng/µl) was targeted to achieve the desired concentration in Treatment 2. The bioassay control dosing solutions used to prepare Treatment 1 consisted of RNase-free water. Dosing solutions were prepared at room temperature.

In addition, positive control dosing solutions containing boric acid were prepared and used to make the positive control diets as described below.

Preparation of Diets

For each diet preparation, the test, bioassay control, and the appropriate positive control dosing solutions were each mixed with carrier in a ratio of approximately 0.75:1 (*i.e.*, 0.75 ml dosing solution to 1 g carrier) generating Treatments 1, 2, 3a, and 3b. After mixing, diets were lyophilized, aliquoted into individual storage tubes, and stored frozen (-80 °C freezer unit) until use.

Sample Collection

Samples of each test dosing solution preparation were collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:12.3 dilution ratio (*e.g.*, 10 µl test dosing solution to 123 µl QHS) on the day of preparation. Samples of Treatment 1 and Treatment 2 were collected into pre-weighed tubes during the process of diet aliquoting as described in Table 44. The tubes containing diet were weighed again in order to obtain the exact sample weight. Samples were stored frozen, if applicable (-80 °C freezer unit), until analysis.

Table 44. Dosing Solution Homogeniety and Stability Results for WCR DvSSJ1 dsRNA Bioassay.

Dosing Solution	Homogeneity in Treatment 2	Stability under Bioassay Conditions in Treatment 2	Frozen Storage Stability in Treatment 2	Absence in Treatment 1
	Number of samples			
Preparation 1	15[a]	25[b]	36[c]	5
Preparation 2	15[a]	NA	NA	5

Note: NA (not applicable).

[a] Five samples were collected from the beginning of the diet aliquoting process, five samples from the middle, and five samples from the end.

[b] Five samples were collected into tubes; the remaining 20 samples were distributed to Petri dishes.

[c] Three samples per time point.

In addition, a portion each of Treatment 1 and Treatment 2 from each diet preparation was collected and stored frozen until use in diets (Treatments A and B, respectively) for sensitive insect bioassays. An individual sensitive insect bioassay was conducted for each diet preparation. Preparation of diets for the sensitive insect bioassays is described in sectionD10.a. Western Corn Rootworm DvSSJ1 dsRNA Sensitive Insect Bioassays . The positive control diets (Treatments 3a and 3b) were not characterized.

Stability under Bioassay Conditions

Verification of stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions was assessed using diet from Preparation 1. For Day 0 of the stability assessment, the five samples collected into pre-weighed tubes were stored frozen (-80 °C freezer unit) until analysis. For the Days 1-4 assessment, samples dispensed on the same day as the Day 0 samples were distributed to Petri dishes along with a water source. Dishes were randomized into stacks containing five samples and placed in an environmental growth chamber set to 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. For each day of stability analysis (Days 1-4), one stack of Petri dishes was removed from the environmental growth chamber. Samples were transferred to individual pre-weighed tubes and stored frozen (- 80 °C freezer unit) until analysis.

Frozen Storage Stability

Samples of Preparation 1 were collected during diet aliquoting for verification of frozen storage stability of DvSSJ1_210 dsRNA in Treatment 2. Three of the 36 samples were designated as Day 0 samples and prepared for analysis. The remaining samples were stored frozen (-80 °C freezer unit). At 11 different time points during the assessment (until after final use of Treatment 2), three samples were removed from the freezer and prepared for analysis.

Preparation of Sample Homogenates

Collected diet samples were removed from the freezer, if applicable, in preparation for extraction and analysis (three each from the beginning, middle, and end of diet aliquoting for homogeneity; three each for Treatment 1 verification of the absence of DvSSJ1_210 dsRNA; three per day for bioassay stability; and three per time point for frozen storage stability).

A volume of QHS equal to 10x sample weight was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were transferred to individual tubes and centrifuged again. The supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS; Treatment 1 samples were loaded undiluted. For frozen storage stability assessment, two of the three samples collected per time point were analyzed. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in each Treatment 2 dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solutions

The concentration of DvSSJ1_210 dsRNA in test dosing solutions was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed for a given diet preparation.

The acceptable range for each diet preparation was calculated using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

All samples were within the acceptable range; therefore, the 95% upper confidence limit (UL) for the probability of a sample falling outside of the acceptable range was calculated using the binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples for each diet preparation was determined to be sufficient to conclude homogeneity.

Verification of Absence of DvSSJ1_210 dsRNA in Treatment 1

Absence of DvSSJ1_210 dsRNA in Treatment 1 samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean MFI of lowest Standard Curve Point – 10%)

Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling day (Days 1 - 4). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

Verification of DvSSJ1_210 dsRNA Frozen Storage Stability in Treatment 2

Frozen storage stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by sampling time point. Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

Demonstration of DvSSJ1_210 dsRNA Activity in WCR Test Diets

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay. Sensitive insect bioassays were performed with *WCR* larvae to demonstrate the biological activity of DvSSJ1_210 dsRNA in Treatment 2. Details regarding the sensitive insect bioassays are provided in section D10.a. Western Corn Rootworm DvSSJ1 dsRNA Sensitive Insect Bioassays

Results and Discussion

Dosing solutions were prepared and incorporated with *Ephestia* eggs to create artificial diets for use in insect feeding studies.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the test dosing solutions and the homogeneity of DvSSJ1_210 dsRNA in the test diets (Treatment 2; Table 45). QuantiGene analysis also verified the absence of DvSSJ1_210 dsRNA in the bioassay control diets (Treatment 1; Table 45). In addition, the stability under bioassay conditions and the frozen storage stability of DvSSJ1_210 dsRNA in Treatment 2 were verified (Table 45, Table 46, and Table 47, respectively).

Sensitive insect bioassays using WCR demonstrated the biological activity of DvSSJ1_210 dsRNA in both Treatment 2 preparations, following storage. Activity was demonstrated by increased mortality and decreased weight of WCR fed diet containing a portion of Treatment 2 when compared to those fed the bioassay control diet containing a portion of Treatment 1. Observed larval mortality and weight data for the WCR sensitive insect bioassays are summarized in Table 48. The WCR bioassays met the acceptability criterion.

Conclusion

Treatments 1, 2, 3a, and 3b were prepared and Treatments 1 and 2 were characterized. All treatments are considered suitable for use in insect feeding studies.

Table 45. Verification of DvSSJ1_210 dsRNA Concentration Assessment in Test Dosing Solutions, Homogeneity in Test Diets and Absence in Bioassay Control Diets for WCR.

Analysis	Preparation	% of Expected	Result
Test Dosing Solution Concentration Verification	1	91	Verified[a]
	2	122	
Homogeneity in Test Diet (Treatment 2)	1	NA	Verified[b]
	2		
Absence in Bioassay Control Diet (Treatment 1)	1	NA	Verified[c]
	2		

Note: Test dosing solutions were collected on the day of dosing solution preparation and diets were collected on the day of diet aliquoting. NA (not applicable).

[a] Concentrations of DvSSJ1_210 dsRNA in test dosing solutions within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified for Treatment 2 if the value of each sample was within 70-130% of the mean value of all samples analyzed for that diet preparation.

[c] Absence of DvSSJ1_210 dsRNA in Treatment 1 was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

Table 46. Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in WCR Test Diet

Dosing Solution Preparation	Bioassay Stability Day	Average % of Day 0 Mean[a]
1	Day 0	NA
	Day 1	103
	Day 2	107
	Day 3	107
	Day 4	111

Note: NA (not applicable).

[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

Table 47. Verification of DvSSJ1_210 dsRNA Frozen Storage Stability in WCR Test Diet

Dosing Solution Preparation	Storage Stability Day	Average % of Day 0 Mean[a]
1	Day 0	NA
	Day 7	108
	Day 18	109
	Day 25	109
	Day 32	100
	Day 40	104
	Day 46	104
	Day 53	112
	Day 61	102
	Day 69	104
	Day 75	114
	Day 82	121

Note: NA (not applicable).

[a] Stored diet samples (-80 °C freezer unit) were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

Table 48. Summary of Sensitive Insect Bioassay Results (for WCR Bioassay)

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1_210 dsRNA/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
Bioassay 1							
A	Bioassay Control Diet	0	30	0	30	2.81 ± 1.19	0.2 - 5.8
B	Test Diet	0.0855	29[a]	89.7	3	0.700 ± 0.700	0.2 - 1.5
Bioassay 2							
A	Bioassay Control Diet	0	30	6.67	28	2.92 ± 1.54	1.0 - 7.4
B	Test Diet	0.0855	30	86.7	4	1.15 ± 0.526	0.7 - 1.9

Note: The sensitive insect bioassay number (1 and 2) corresponds to the dosing solution preparation used to prepare Treatments 1 and 2. Treatments A and B were prepared from the same diet preparations used in Treatments 1 and 2, respectively. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

[a] Organisms counted as missing or lost in transfer during a bioassay were not included in the total number of observations for a given treatment.

D10.a. Western Corn Rootworm DvSSJ1 dsRNA Sensitive Insect Bioassays

The biological activity of DvSSJ1_210 dsRNA in stored test diet (Treatment 2) from each diet preparation was evaluated by conducting individual 14-day bioassays using WCR, a species sensitive to DvSSJ1_210 dsRNA. Each WCR bioassay was initiated near final use of the test diet in insect feeding bioassays to demonstrate the DvSSJ1_210 dsRNA remained biologically active after diets had been stored frozen.

WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassays consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

For each WCR bioassay, larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)
- Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.0855 ng DvSSJ1_210 dsRNA per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals for each bioassay.

Diet Preparation

For each dosing solution preparation, stored samples (-80 °C freezer unit) of Treatments 1 and 2 were used to prepare Treatments A and B for an individual WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1, resulting in a 30% incorporation of Treatment 1 by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 2, resulting in a 30% incorporation of Treatment 2 by dry weight of the WCR diet.

For each respective treatment, RNase-free water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassays

For each bioassay, WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used within 24 hours of hatching. On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were

dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. Each bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every three to four days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, each bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well or lost in transfer were excluded from statistical analysis.

The bioassay acceptability criteria indicated a bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for each sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 48. Weight data were summarized as means, standard deviations, and ranges and are presented in Table 48.

D11. Evaluation of the Survival and Development of Pink Spotted Lady Beetle Fed an Artificial Diet Containing DvSSJ1 210 bp ds RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of decapsulated brine shrimp (*Artemia* sp.) eggs.

Positive Control

The positive control consisted of cryolite (AlF_6Na_3).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solution used to prepare Treatment 2 consisted of the test substance diluted in RNase-free water to achieve the concentration in the test diet.

Test System

The test system was *Coleomegilla maculata* (pink spotted lady beetle; Coleoptera: Coccinellidae; CMAC). CMAC was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. CMAC eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

CMAC larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 10,000 ng cryolite per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a stack of six Petri dishes secured together and contained 2 replicates from each treatment. Each treatment was fed to a target of 30 CMAC individuals. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a

photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 20, all organisms had either died or emerged and the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity, frozen storage stability, and stability under bioassay conditions of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

Bias in the CMAC bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria.

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1 and 2 were prepared as described in section D11.a. CMAC DvSSJ1 dsRNA Bioassay Analytical Phasebelow. Each dosing solution was mixed with carrier in a 1:1 ratio (*i.e.*, 1 g carrier to 1 ml dosing solution). Treatment 3 was prepared by mixing cryolite with carrier to a nominal concentration of 10,000 ng cryolite per mg carrier dry weight and then combining with RNase-free water in a 1:1 ratio. After mixing, diets were lyophilized, aliquoted into individual storage tubes, and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1 and 2 were collected for characterization of diets as described in section D11.a. CMAC DvSSJ1 dsRNA Bioassay Analytical Phase. In addition, a portion each of Treatment 1 and Treatment 2 was collected during the process of diet aliquoting for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in sectionD11.b. CMAC DvSSJ1 dsRNA Sensitive Insect Bioassay.

The positive control diet (Treatment 3) was not characterized.

***CMAC* Bioassay**

CMAC eggs were incubated in an environmental chamber until the eggs hatched. CMAC neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet aliquots were removed from frozen storage and approximately 20 mg of diet were aliquoted into individual caps. A cap containing the appropriate diet treatment and a moisture source (a tube filled with 0.5% agar) were distributed to each Petri dish utilized in the bioassay. One CMAC neonate was placed in each dish and the dishes were stacked into blocks and secured together. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Every 3-4 days, dishes were removed from the environmental chamber, pupation was assessed, and missing or dead organisms were recorded. For each living organism that had not yet pupated or died, old diet was removed from the dish and a new diet-filled cap was prepared and distributed as described for Day 0, prior to returning the dishes to the environmental chamber. Once pupation was observed, organisms were assessed daily for adult emergence. CMAC adults were weighed within approximately 24 hours of emergence. The bioassay was complete when all larvae had either died or emerged as adults and final mortality was assessed. Only dishes that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a dish were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality, weight, and days to adult emergence. A statistical comparison was made between CMAC fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

CMAC fed the artificial diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of CMAC fed the artificial diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatment 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Number of Days to Adult Emergence

The normality assumption necessary for a two-sample *t*-test was not satisfied by data distributions of treatment groups 1 and 2; therefore, a non-parametric two-sample test was conducted to examine if exposure to DvSSJ1_210 dsRNA caused developmental delay. The corresponding hypothesis test was

$$H_0 : F_T(x) = F_c(x) \ \ vs. \ \ H_a : F_T(x) > F_c(x)$$

Where $F_T(x)$ is the cumulative distribution of the number of days to adult emergence of CMAC fed the artificial insect diet containing DvSSJ1_210 dsRNA, and $F_c(x)$is the cumulative distribution of the number of days to adult emergence of individuals fed the bioassay control diet. Therefore, significance would indicate that insects fed the diet containing DvSSJ1_210 dsRNA would have a greater probability to take longer to emerge than those fed the control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Demonstration of DvSSJ1_210 dsRNA Activity in the *CMAC* Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay. A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the DvSSJ1_210 dsRNA used in Treatment 2 in the CMAC

bioassay. Details regarding the sensitive insect bioassay are provided in section D11.b. CMAC DvSSJ1 dsRNA Sensitive Insect Bioassay.

Results and Discussion

The CMAC bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 49.

The mortality of CMAC fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 10.3%; P-value = 0.1124) was not significantly greater than those fed the bioassay control diet (Treatment 1; 0%), as shown in Table 49.

The mean weight of CMAC fed Treatment 2 (13.0 mg; P-value = 0.7611) was not significantly less than the mean weight of those fed Treatment 1 (12.6 mg), as shown in Table 50.

CMAC fed Treatment 2 (median 14 days; Wilcoxon test P-value = 0.9429) and Treatment 1 (median 15 days) did not significantly differ in the probability to take longer to emerge, as shown in Table 51. In addition, the Siegel-Tukey test (P-value = 0.2679) did not show significant evidence that the scales of the two populations differed.

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 52. The WCR bioassay met the acceptability criterionD11.b. CMAC DvSSJ1 dsRNA Sensitive Insect Bioassay). The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 of the *CMAC* bioassay was demonstrated by increased mortality and decreased weight of *WCR* fed the test diet (Treatment B; described in Table 52 and section D11.b. CMAC DvSSJ1 dsRNA Sensitive Insect Bioassay) when compared to the bioassay control diet.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity of DvSSJ1_210 dsRNA in Treatment 2 (Table 53).

The absence of DvSSJ1_210 dsRNA in Treatment 1 was verified in one sample but trace amounts were found in the other four samples (Table 53). Treatment 1 samples were analyzed undiluted while Treatment 2 samples were diluted 1:16000 for analysis. If diluted to the same level as Treatment 2 samples, the DvSSJ1_210 dsRNA signal in the affected Treatment 1 samples would not have been detectable and it is estimated that the signal would have been 10,000-30,000 times less than in Treatment 2 based on the results of each Treatment 1 sample. Although there were trace amounts of DvSSJ1_210 dsRNA detected in the bioassay control diet, results for CMAC mortality, weight, and days to adult emergence in this study compared to historical data indicate no effect on the bioassay control diet group response and no impact on this study.

In addition, the frozen storage stability (over 41 days in a -80 °C freezer unit) and stability under bioassay conditions (over four days) of DvSSJ1_210 dsRNA in Treatment 2 was verified Table 54). Although diets stored under bioassay conditions were slightly under the acceptability criterion on Day 2, diets stored under bioassay conditions did meet the acceptability criterion on Days 1, 3, and 4 (Table 54).

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival, weight, or adult emergence of CMAC.

Table 49. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	1	29[a]	3	10.3	0.1124
3	Positive Control Diet	0[b]	28[a]	28	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

Table 50. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	12.6 (11.7 - 13.5)	7.8 - 18.0	--
2	Test Diet	1	26	13.0 (12.3 - 13.7)	9.2 - 16.8	0.7611
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving *CMAC* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

Table 51. Summary Analysis of CMAC DvSSJ1 dsRNA Bioassay Days to Adult Emergence Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	30	15.0 ± 1.17	15	14 - 20	--	--
2	Test Diet	1	26	14.6 ± 0.983	14	13 - 17	0.9429	0.2679
3	Positive Control Diet	0[a]	0	NA	NA	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving *CMAC* in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

Table 52. Summary of CMAC DvSSJ1 dsRNA Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	29[a]	6.90	27	2.87 ± 1.25	0.6 - 5.9
B	Test Diet	0.0855	30	80.0	6	0.500 ± 0.352	0.3 - 1.2

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the *CMAC* bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 53. Assessment of DvSSJ1_210 dsRNA Concentration in CMAC Test Dosing Solution, Homogeneity in Test Diet and Absence in Bioassay Control Diet

Analysis	Time Point	Diet Sample	Result
Test Dosing Solution Concentration Verification	Day of Dosing Solution Preparation	NA	Verified[a]
Homogeneity in Test Diet (Treatment 2)	Day of Diet Aliquoting	Beginning	Verified
		Middle	
		End	
Absence in Bioassay Control Diet (Treatment 1)	Day of Diet Aliquoting	First	Verified
		Second	Trace[b]
		Third	
		Fourth	
		Fifth	

Note: Not applicable (NA). Beginning, middle, and end refer to the stages in the diet aliquoting process at which homogeneity samples were collected.

[a] The test dosing solution concentration was 102% of the expected DvSSJ1_210 dsRNA concentration. Concentrations within 70-130% of the expected value were considered verified.

[b] Bioassay control diet (Treatment 1) samples were loaded undiluted; test diet (Treatment 2) samples were diluted 1:16,000 for analysis. If diluted to the same level as Treatment 2 samples, the DvSSJ1_210 dsRNA signal in the affected Treatment 1 samples would not have been detectable and it is estimated that the signal would have been 10,000-30,000 times less than in Treatment 2.

Table 54. Verification of DvSSJ1_210 dsRNA Stability in CMAC Test Diet

Stability under Bioassay Conditions (Treatment 2)		Frozen Storage Stability (Treatment 2)	
Bioassay Stability Day	Average % of Day 0 Mean[a]	Storage Day	Average % of Day 0 Mean[a]
Day 0	NA	0	NA
Day 1	81	11	98
Day 2	69	21	102
Day 3	73	28	112
Day 4	73	34	118
		41	116

Note: Frozen storage stability samples were stored in a -80 °C freezer unit. Not applicable (NA).

[a] Diet samples were considered stable if the average percentage for each sampling time point and condition was ≥ 70% of the Day 0 mean.

D11.a. CMAC DvSSJ1 dsRNA Bioassay Analytical Phase

The following DvSSJ1_210 dsRNA and control dosing solutions were prepared for the CMAC bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water

Test dosing solution used to prepare Treatment 2 consisting of test substance diluted in RNase-free water to achieve the concentration in the test diet

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity, frozen storage stability, and stability under bioassay

conditions of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Dosing solutions were prepared on the day of diet aliquoting for the CMAC bioassay. To generate the test dosing solution for Treatment 2, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in RNase-free water to the appropriate DvSSJ1_210 dsRNA concentration (1 ng/µl). The bioassay control dosing solution consisted of RNase-free water. Dosing solutions were maintained chilled until use.

Sample Collection

A sample of the test dosing solution was collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:10 dilution (e.g.; 10 µl test dosing solution to 90 µl QHS) on the day of preparation. Samples of Treatments 1 and 2 were collected into pre-weighed tubes during the process of diet aliquoting for the CMAC bioassay as described in Table 55. The tubes containing diet were weighed again in order to obtain the exact sample weight. Samples were stored frozen if applicable (-80 °C freezer unit) until analysis.

Table 55. Test Dosing Solutions and Analyses for CMAC DvSSJ1 dsRNA Bioassays

Treatment	Number of Samples	Analysis
1	5	Verify absence of DvSSJ1_210 dsRNA
2	5 beginning	Homogeneity of DvSSJ1_210 dsRNA
	5 middle	
	5 end	
	25[a]	Stability under bioassay conditions
	18 (3 each time point)	Frozen storage stability (-80 °C freezer unit)

Note: Beginning, middle, and end refer to the stage of the diet distribution process at which samples were collected.

[a] Five samples were collected into tubes; the remaining samples were distributed to Petri dishes and placed under bioassay conditions.

Stability under Bioassay Conditions

Samples were collected during the process of diet aliquoting for verification of stability of DvSSJ1_210 dsRNA in Treatment 2 under bioassay conditions. For the Day 0 assessment, five samples were collected into pre-weighed tubes and stored frozen (-80 °C freezer unit) until analysis. For Days 1-4 assessment, samples (five per day) were distributed to individual Petri dishes along with a water source, as described for the *CMAC* bioassay, except the dishes were not infested with larvae. Dishes were organized into stacks and placed under bioassay conditions using the same methods and conditions used in the *CMAC* bioassay. For each day of stability analysis (Days 1-4), one stack of Petri dishes containing five samples of Treatment 2 was removed from bioassay conditions. The diet in each dish was transferred to an individual pre-weighed tube, and the tube containing diet sample reweighed before being stored frozen (-80 °C freezer unit) until analysis.

Preparation of Sample Homogenates

Diet samples were removed from the freezer in preparation for extraction and analysis (three each from the beginning, middle, and end of diet aliquoting for homogeneity samples, five for Treatment 1 verification of the absence of DvSSJ1_210 dsRNA, three each for Day 0-4 bioassay stability, and three per time point for frozen storage stability).

A volume of QHS (µl) equal to 10x sample weight (mg) was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were transferred to individual tubes and centrifuged again. The supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), thawed and warmed, and then diluted as necessary in QHS. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was

proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentrations of DvSSJ1_210 dsRNA in the Treatment 2 dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solution

The concentration of DvSSJ1_210 dsRNA in test dosing solution was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed.

The acceptable range was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Verification of DvSSJ1_210 dsRNA Stability under Bioassay Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by time point (Days 1 - 4). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

Verification of DvSSJ1_210 dsRNA Stability under Frozen Storage Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by time point (Days 11, 21, 28, 34, and 41). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

D11.b. CMAC DvSSJ1 dsRNA Sensitive Insect Bioassay

The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 used in the CMAC (CMAC) bioassay was evaluated by conducting a 14-day bioassay using WCR, a species sensitive to DvSSJ1_210 dsRNA. The WCR bioassay was initiated after completion of the CMAC bioassay.

WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)
- Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.0855 ng DvSSJ1_210 dsRNA per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 2 for the CMAC bioassay were removed from the freezer and used to prepare Treatments A and B for each day of diet preparation for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the CMAC bioassay, resulting in a 30% incorporation of the CMAC diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 2 from the CMAC bioassay, resulting in a 30% incorporation of the CMAC diet by dry weight of the WCR diet.

For each respective treatment, RNase-free water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 52. Weight data were summarized as means, standard deviations, and ranges and are also presented in Table 52.

D12. Evaluation of the Survival of Rove Beetle Fed an Artificial Diet Containing DvSSJ 210 bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of *Ephestia* (*Ephestia kuehniella* sp.) eggs.

Test System

The test system was *Dalotia coriaria* (rove beetle; Coleoptera: Staphylinidae; RVB). RVB was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. RVB adults were obtained from Beneficial Insectary, Inc. (Redding, CA, USA) and identity was recorded by study personnel.

Experimental Design

RVB adults were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 20,000 ng boric acid per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 3 blocks. Each block consisted of 1-oz plastic cups in a 30-well tray containing 10 replicates from each treatment. Each treatment was fed to a target of 30 RVB individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Adults were refed daily. After 14 days, the bioassay was complete and mortality was assessed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared, aliquoted, and characterized under a separate study (see section D10. Preparation and Characterization of an Artificial Diet Containing *Ephestia* Eggs Incorporated with DvSSJ1 210bp ds RNA). A certificate of analysis is included in the study records. The positive control diet (Treatment 3) was also prepared under a separate study (D10. Preparation and Characterization of an Artificial Diet Containing *Ephestia* Eggs Incorporated with DvSSJ1 210bp ds RNA) but was not characterized.

RVB Bioassay

A bioassay was conducted to determine the response of RVB to DvSSJ1_210 dsRNA exposure via oral ingestion. RVB adults were incubated with a water source but no food for at least 24 hours prior to initiation of the bioassay.

On Day 0 of the bioassay, diet aliquots were removed from frozen storage and trays were prepared by placing plastic cups in each tray. A moisture source (0.5% agar solution) was added to each plastic cup utilized in the bioassay and the agar was allowed to cool. Individual Post-it® flags were coated with diet from a single treatment. The diet-coated flags were cut into a maximum of 10 rectangular aliquots of approximately equal size and distributed to each plastic cup containing agar. One RVB adult was placed in each cup containing diet, and then the cup was sealed with a lid. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for 14 days. Trays were removed from the environmental chamber and missing or dead organisms were recorded daily. Diet coated flags were replaced with new aliquots as described for Day 0 and the trays were returned to the environmental chamber. After 14 days, the bioassay was complete and mortality was assessed.

Statistical Analysis

Statistical comparison for mortality was made between RVB fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) using SAS software, Version 9.4.

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of RVB fed the artificial diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0$.

A significant difference was identified if the P-value was <0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Results and Discussion

The RVB bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 56.

The RVB bioassay was run twice. On the initial run, mortality in Treatment 1 did not meet the acceptability criteria, likely due to an insufficient amount of agar provided to the organisms as a moisture source. The bioassay was repeated with an increased amount of agar dispensed to each organism and using freshly prepared dosing solutions and diets. The second run of the bioassay met the acceptability criterion; therefore, results for the second run of the RVB bioassay are included in this report. Data generated for the initial run of the RVB bioassay are not included in this report, but were retained with the study records.

The mortality of RVB fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 0%; P-value = 1.0000) was not significantly greater than those fed the bioassay control diet (Treatment 1; 3.33%), as shown in Table 56.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival of RVB.

Table 56. Summary Analysis of RVB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	1	30	0	0	1.0000
3	Positive Control Diet	0 [a]	30	30	100	--

Note: Targeted DvSSJ1_210 dsRNA concentrations in Treatment 2 were based on diet dry weight.
[a] Treatment 3 contained a targeted concentration of 20,000 ng boric acid per mg diet dry weight.

D13. Evaluation of the Survival and Weight of European Corn Borer Fed Artificial Diet Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of Stonefly Heliothis diet.

Test System

The test system was *Ostrinia nubilalis* (European corn borer; Lepidoptera: Crambidae; ECB). ECB was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. ECB eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

ECB larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 3750 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a 6-well bioassay plate and contained 2 replicates from each treatment. Each treatment was fed to a target of 30 ECB individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared and characterized under a separate study (see section D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA). The positive control diet (Treatment 3) was also prepared under the same study but not characterized.

ECB Bioassay

ECB eggs were incubated in an environmental chamber until the eggs hatched. ECB neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One ECB neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0 with the exception that on Day 7, 600 µl was dispensed per well and on Day 11, 1000 µl was dispensed per well. The additional diet was needed to accommodate the increased nutritional needs of the growing organisms. Living ECB larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4. The response variables of interest were mortality and weight. A statistical comparison was made between ECB fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

ECB fed the artificial diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of ECB fed the artificial diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatments 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Results and Discussion

The ECB bioassay was run twice. On the initial run, the bioassay did not meet the acceptability criteria as the number of dead and missing larvae in the bioassay control diet (Treatment 1) exceeded 20%. The bioassay was re-run using freshly prepared dosing solutions and diets and the second run met the acceptability criteria; therefore, results for the second run are included in this report. Data generated for the initial run of the bioassay were not included in this report, but were retained with the study records.

The mortality of ECB fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 10.0%; P-value = 0.5165) was not significantly greater than those fed the bioassay control diet (Treatment 1; 6.90%), as shown in Table 57.

The mean weight of ECB fed Treatment 2 (107 mg; P-value = 0.8843) was not significantly less than those fed Treatment 1 (98.2 mg), as shown in Table 58.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or weight of ECB.

Table 57. Summary Analysis of ECB DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	29[a]	2	6.90	--
2	Test Diet	1	30	3	10.0	0.5165
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 3750 ng boric acid per mg diet wet weight.

Table 58. Summary Analysis of ECB DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	27	98.2 (86.5 - 110)	7.7 - 141.9	--
2	Test Diet	1	27	107 (97.6 - 117)	33.7 - 153.1	0.8843
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving ECB in the positive control diet group.

[a] Treatment 3 contained a targeted concentration of 3750 ng boric acid per mg diet wet weight.

D14. Evaluation of the Survival and Weight of Corn Earworm Fed Artificial Diet Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of Stonefly Heliothis diet.

Test System

The test system was *Helicoverpa zea* (corn earworm; Lepidoptera: Noctuidae; CEW). CEW was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. CEW eggs were obtained from Benzon Research Inc. (Carlisle, PA, USA) and identity was confirmed by study personnel.

Experimental Design

CEW larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 3125 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a 6-well bioassay plate and contained 2 replicates from each treatment. Each treatment was fed to a target of 30 CEW individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 12 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared and characterized under a separate study (see section D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA). A certificate of analysis is included in the study records. The positive control diet (Treatment 3) was also prepared under the same study but not characterized.

***CEW* Bioassay**

CEW eggs were incubated in an environmental chamber until the eggs hatched. CEW neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One CEW neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 12 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0 with the exception that on Day 4, 1500 µl was dispensed per well and on Day 7 and Day 11, 3000 µl was dispensed per well. The additional diet was needed to accommodate the increased nutritional needs of the growing organisms. Living CEW larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. During the Day 7 refeed and CEW transfer, two bioassay plates did not receive holes for ventilation and those organisms died. Mortality for those organisms was reported in the study records as a transfer error and the organisms were excluded from statistical analysis. After 12 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4. The response variables of interest were mortality and weight. A statistical comparison was made between CEW fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of CEW fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of CEW fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatments 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Results and Discussion

The CEW bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 59.

The mortality of CEW fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 7.69%; P-value = 0.6951) was not significantly greater than those fed the bioassay control diet (Treatment 1; 7.69%), as shown in Table 59.

The mean weight of CEW fed Treatment 2 (396 mg; P-value = 0.3372) was not significantly less than those fed Treatment 1 (408 mg), as shown in Table 60.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2) had no adverse effect on survival or weight of CEW.

Table 59. Summary Analysis of CEW DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	26[a]	2	7.69	--
2	Test Diet	1	26[a]	2	7.69	0.6951
3	Positive Control Diet	0[b]	28[ac]	26	92.9	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] A transfer error killed four organisms each in Treatments 1 and 2 and one organism in Treatment 3. Those organisms were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 3125 ng boric acid per mg diet wet weight.

[c] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 60. Summary Analysis of CEW DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	24	408 (363 - 453)	45.9 - 573.0	--
2	Test Diet	1	24	396 (362 - 431)	81.2 - 513.6	0.3372
3	Positive Control Diet	0[a]	2	0.750 ± 0.636[b]	0.3 - 1.2	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Treatment 3 contained a targeted concentration of 3125 ng boric acid per mg diet wet weight.

[b] Standard deviation is provided for mean values not subjected to a *t*-test.

D15. Evaluation of the Survival and Weight of Painted Lady Fed Artificial Diet Containing DvSSJ1 210bp ds RNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of Stonefly Heliothis diet.

Test System

The test system was *Vanessa cardui* (painted lady; Lepidoptera: Nymphalidae; PL). PL was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. PL eggs were obtained from Carolina Biological Supply Company (Burlington, NC, USA) and identity was recorded by study personnel.

Experimental Design

PL larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 2500 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a 6-well bioassay plate and contained 2 replicates from each treatment. Each treatment was fed to a target of 30 PL individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared and characterized under a separate study (see section D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA). The positive control diet (Treatment 3) was also prepared under the same study but was not characterized.

PL Bioassay

PL eggs were incubated in an environmental chamber until the eggs hatched. PL neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One PL neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark for a total of 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0 with the exception that on Day 4, 600 µl was dispensed per well; on Day 7, 1500 µl was dispensed per well; and on Day 11, 2000 µl was dispensed per well. The additional diet was needed to accommodate the increased nutritional needs of the growing organisms. Living PL larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4. The response variables of interest were mortality and weight. A statistical comparison was made between PL fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

PL fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of PL fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatments 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Results and Discussion

The PL bioassay met the acceptability criteria as the number of dead and missing larvae in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 61.

The mortality of PL fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 26.7%; P-value = 0.3805) was not significantly greater than those fed the bioassay control diet (Treatment 1; 20.0%), as shown in Table 61. The mean weight of PL fed Treatment 2 (405 mg; P-value = 0.6635) was not significantly less than those fed Treatment 1 (391 mg), as shown in Table 62. The observed difference in mortality between Treatment 2 and Treatment 1 is not considered biologically relevant in this bioassay, given the mean weight and range of weights.

Conclusion

The results demonstrated the mortality for PL fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 26.7%) was not significantly greater than the mortality for PL fed the bioassay control diet (Treatment 1; 20.0%). The mean weight of PL fed Treatment 2 (405 mg) was not significantly less than the mean weight of PL fed Treatment 1 (391 mg).

Table 61. Summary Analysis of PL DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	6	20.0	--
2	Test Diet	1	30	8	26.7	0.3805
3	Positive Control Diet	0[a]	28[b]	27	96.4	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Treatment 3 contained a targeted concentration of 2500 ng boric acid per mg diet wet weight.
[b] Due to transfer error, two organisms were not included in mortality calculations for Treatment 3.

Table 62. Summary Analysis of PL DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	24	391 (336 - 445)	86.7 - 566.4	--
2	Test Diet	1	22	405 (364 - 445)	226.7 - 565.3	0.6635
3	Positive Control Diet	0[a]	1	1.40	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there was only one surviving PL in the positive control diet group.
[a] Treatment 3 contained a targeted concentration of 2500 ng boric acid per mg diet wet weight.

D16. Evaluation of the Survival and Weight of Codling Moth Fed Artificial Diet Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. The stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of Stonefly Heliothis diet.

Test System

The test system was *Cydia pomonella* (codling moth; Lepidoptera: Tortricidae; CDM). CDM was selected as a representative lepidopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. CDM eggs were obtained from Benzon Research Inc. (Carlisle, PA, USA) and identity was recorded by study personnel.

Experimental Design

CDM larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 5000 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained 3 replicates from each treatment. Each treatment was fed to a target of 30 CDM individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2, respectively) were prepared and characterized under a separate study (see section D5. Preparation and Characterization of Stonefly Heliothis Artificial Diet Incorporated with DvSSJ1 210bp dsRNA). The positive control diet (Treatment 3) was also prepared under the same study but was not characterized. On Day 7, the amount of boric acid used was incorrect, increasing the concentration of boric acid in Treatment 3.

CDM Bioassay

CDM eggs were incubated in an environmental chamber until the eggs hatched. CDM neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One CDM neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark for a total of 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0 with the exception that on Day 11, 600 µl was dispensed per well. The additional diet was needed to accommodate the increased nutritional needs of the growing organisms. Living CDM larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparison was made between CDM fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher’s exact test to determine if the mortality rate of

CDM fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition; *i.e.*, lower weight of CDM fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test is

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatments 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Results and Discussion

For the CDM bioassay (Table 63), the positive control diet (Treatment 3) group met the acceptability criteria as mortality exceeded 80%. The bioassay control diet (Treatment 1) group did not meet the acceptability criterion as the combined dead and missing CDM count exceeded 20%; however, as mortality alone was 11.5% on Day 14, the bioassay was not repeated. Between Day 11 (mortality plus missing = 10.0%) and Day 14 (mortality plus missing = 23.3%), a number of organisms across treatments managed to eat through the lidding material. These organisms were marked as missing on the data sheets but were physically able to chew out of their wells, indicating they were alive at that time.

The mortality of CDM fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 8.00%; P-value = 0.8129) was not significantly greater than those fed the bioassay control diet (Treatment 1; 11.5%), as shown in Table 63.

The mean weight of CDM fed Treatment 2 (54.2 mg; P-value = 0.2979) was not significantly less than those fed Treatment 1 (56.5 mg), as shown in Table 64.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2) had no adverse effect on survival or weight of CDM.

Table 63. Summary Analysis of CDM DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	26[a]	3	11.5	--
2	Test Diet	1	25[a]	2	8.00	0.8129
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Treatment 3 contained a targeted concentration of 5000 ng boric acid per mg diet wet weight.

Table 64. Summary Analysis of CDM DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	23	56.5 (50.3 - 62.6)	20.8 - 82.9	--
2	Test Diet	1	23	54.2 (48.0 - 60.5)	3.4 - 77.2	0.2979
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight. Not applicable (NA); there were no surviving CDM in the positive control diet group.

[a] Treatment 3 contained a targeted concentration of 5000 ng boric acid per mg diet wet weight.

Appendix E. DvSSJ1 dsRNA Non-Target Organism Bioassay Materials and Methods

E1. Evaluation of the Survival and Reproduction of Springtail Fed an Artificial Diet Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of ground and autoclaved *Saccharomyces cerevisiae* (Type II) yeast.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solution used to prepare Treatment 2 consisted of the test substance diluted in RNase-free water to achieve the concentration in the test diet.

The positive control dosing solution used to prepare Treatment 3 consisted of teflubenzuron ($C_{14}H_6Cl_2F_4N_2O_2$) and ethanol.

Test System

The test system was *Folsomia candida* (springtail; Collembola: Isotomidae). Springtail was selected as a representative non-target organism to characterize DvSSJ1_210 dsRNA. Springtail adults were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

Springtail adults were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 1000 ng teflubenzuron per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 2 blocks. Each block consisted of 12 small, wide-mouth glass jars (four jars per treatment) prepared with substrate and topped with loosely-capped screw top lids. Each jar contained a target of 10 adult organisms and each treatment was fed to a target of 80 Springtail individuals. The bioassay was conducted in an environmental chamber set at 20 °C, 65% relative humidity, and continuous dark. Springtail adults and any resulting offspring were refed daily. After 28 days, the bioassay was complete, adult mortality was assessed, and adult and juvenile organisms were each counted.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- Adult mortality exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mean offspring per bioassay vessel is less than 100 individuals for Treatment 1.
- The coefficient of variation (CV) for mean reproduction is greater than 30% for Treatment 1.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity and frozen storage stability of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

Bias in the Springtail bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria.

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1 and 2 were prepared as described below. The positive control dosing solution for Treatment 3 was prepared by solubilizing and diluting teflubenzuron in ethanol to achieve a nominal concentration of 1000 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 2:1 ratio (*i.e.*, 2 ml dosing solution to 1 g carrier) to generate Treatments 1-3. After mixing, diets were lyophilized, aliquoted into individual storage tubes, and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1 and 2 were collected for characterization of diets as described in section E1.a. Springtail DvSSJ1 dsRNA Bioassay Analytical Phase. In addition, a portion each of Treatment 1 and Treatment 2 was collected for use in diets for the sensitive insect bioassay and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in sectionE1.b. Springtail DvSSJ1 dsRNA Sensitive Insect Bioassay.

The positive control diet (Treatment 3) was not characterized.

Springtail Bioassay

A bioassay was conducted to determine the response of Springtail to DvSSJ1_210 dsRNA exposure via oral ingestion.

On Day 0, diet aliquots were removed from storage, glass jars were prepared with a substrate mixture containing 47% Plaster of Paris, 6% charcoal, and 47% deionized water, and substrate was allowed to dry. Bioassay jars were labeled by treatment, replicate, and study number and a target of ten, 11-day-old Springtail adults were placed in each jar. Diets were distributed to small squares of weigh paper by treatment, a weigh paper square was placed in each infested bioassay jar, and the jars were loosely capped. The bioassay was conducted in an environmental chamber set at 20 °C, 65% relative humidity, and continuous dark. Every day during the bioassay, infested jars were removed from the environmental chamber, diet was replaced with new aliquots as described for Day 0, dry substrate was re-saturated with deionized water as necessary, and the bioassay jars were loosely capped and returned to the environmental chamber. After 28 days, the bioassay was complete, adult mortality was assessed, and adult and juvenile organisms were each counted.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and reproduction. Statistical comparisons were made between Springtail fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for the response variables.

Mortality

Statistical analysis was conducted using Fisher's exact test to compare if the mortality rate of Springtail fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was higher than the

mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

Significance was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct Fisher's exact test.

Reproduction

A generalized linear mixed model was fit to the reproduction data assuming a Poisson distribution of the number of offspring, a log link function, and the Laplace method of integral approximation. Treatment was modeled as a fixed effect. Jar numbers within each treatment and block were considered random effect.

The estimated model was used to test if the reproduction from the adults fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) was less than the reproduction from the adults fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

SAS PROC GLIMMIX was utilized for generalized linear mixed model analysis, and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Demonstration of DvSSJ1_210 dsRNA Activity in the Springtail Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay using WCR larvae to demonstrate the biological activity of the DvSSJ1_210 dsRNA used in Treatment 2 in the Springtail bioassay, as described in sectionE1.b. Springtail Sensitive Insect Bioassay**.**

Results and Discussion

The Springtail bioassay met the acceptability criteria as adult mortality in the bioassay control diet (Treatment 1) group did not exceed 20%, the mean offspring count in Treatment 1 was ≥ 100, the CV for mean reproduction in Treatment 1 was ≤ 30%, and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 65 and Table 66.

The mortality of Springtail fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 0%; P-value = 1.0000) was not significantly greater than those fed the bioassay control diet (Treatment 1; 3.75%), as shown in Table 65.

The mean reproduction of Springtail fed Treatment 2 (345 offspring; P-value=0.8290) was not significantly less than the mean reproduction of those fed Treatment 1 (319 offspring), as shown in Table 66.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the Treatment 2 test dosing solution and the homogeneity and frozen storage stability of DvSSJ1_210 dsRNA in Treatment 2, as shown in Table 69 and Table 70. The absence of DvSSJ1_210 dsRNA in Treatment 1 was also verified, as shown in Table 69.

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 67 and Table 68. The WCR bioassay met the acceptability criterion. The mortality of WCR fed the test diet (Treatment B; 50.0%; P-value = 0.1102) was not significantly different from those fed the bioassay control diet (Treatment A; 26.7%), as shown in Table 67. The mean weight of WCR fed Treatment B (1.20 mg; P-value <0.0001) was significantly different from those fed Treatment A (2.88 mg), as shown in Table 68. The dose in the sensitive insect test diet, 0.057 ng DvSSJ1_210 dsRNA per mg, yielded expected results based on WCR sensitivity (Appendix D, Section D1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing DvSSJ1_210 double stranded RNA). The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 of the Springtail bioassay was demonstrated by decreased weight of WCR fed the test diet when compared to the bioassay control diet.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival or reproduction of Springtail.

Table 65. Summary Analysis of Springtail DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	80	3	3.75	--
2	Test Diet	1	81[a]	0	0	1.0000
3	Positive Control Diet	0[b]	80	77	96.3	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.

[a] One jar for Treatment 2 contained 11 organisms.

[b] Treatment 3 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

Table 66. Summary Analysis of Springtail DvSSJ1 dsRNA Bioassay Reproduction Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Mean Number of Offspring (95% Confidence Interval)	Range (mg)	CV	P-Value
1	Bioassay Control Diet	0	319 (283-360)	268 - 399	14.5	--
2	Test Diet	1	345 (306-389)	264 - 462	19.6	0.8290
3	Positive Control Diet	0[a]	0	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no offspring recorded forSpringtail in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

Table 67. Summary Analysis of Springtail DvSSJ1 dsRNA Sensitive Insect Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
A	Bioassay Control Diet	0	30	8	26.7	--
B	Test Diet	0.057	30	15	50.0	0.1102

Note: Treatments A and B used in the sensitive insect (WCR) bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the *Folsomia candida* bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

Table 68. Summary Analysis of Springtail DvSSJ1 dsRNA Sensitive Insect Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)		P-Value
				Mean (95% Confidence Interval)	Range	
A	Bioassay Control Diet	0	22	2.88 (2.44 - 3.33)	0.6 - 5.3	--
B	Test Diet	0.057	15	1.20 (0.737 - 1.66)	0.3 - 2.8	<0.0001[a]

Note: Treatments A and B used in the sensitive insect (WCR) bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the Springtail bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

[a] A statistically significant difference (P-Value <0.05) was observed.

Table 69. Verification of DvSSJ1_210 dsRNA Concentration in Test Dosing Solution, Homogeneity in Test Diet, and Absence in Bioassay Control Diet for Springtail Bioassay.

Analysis	Diet Sample	Result
Test Dosing Solution Concentration Verification	N/A	Verified[a]
Homogeneity in Test Diet (Treatment 2)	Beginning	Verified[b]
	Middle	
	End	
Absence in Bioassay Control Diet (Treatment 1)	First	Verified[c]
	Second	
	Third	

[a]The test dosing solution concentration was 129% of the expected DvSSJ1_210 dsRNA result. Concentrations within 70-130% of the expected value were considered verified.
[b]Homogeneity was considered verified if the value of each sample was within 70-130% of the mean value of all samples analyzed.
[c] Absence of DvSSJ1_210 dsRNA in Treatment 1 was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

Table 70. Verification of Frozen Storage Stability of DvSSJ1_210 dsRNA in Test Diet (Treatment 2) for Springtail Bioassay.

Storage Day	Average % of Day 0 Mean[a]
0	NA
8	104
15	97
23	103
29	113
37	104
44	106

Note: Frozen storage stability samples were stored in a -80 °C freezer unit. Not applicable (NA).
[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

E1.a. Springtail DvSSJ1 dsRNA Bioassay Analytical Phase

The following dosing solutions were prepared for the Springtail bioassay:

- Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water
- Test dosing solution used to prepare Treatment 2 consisting of test substance diluted in RNase-free water to achieve the concentration in the test diet

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity and frozen storage stability of DvSSJ1_210 dsRNA in the

test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also verified. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation for the Springtail bioassay. To generate the test dosing solution for Treatment 2, the test substance was removed from frozen storage, allowed to thaw at room temperature, and then diluted in RNase-free water to the appropriate DvSSJ1_210 dsRNA concentration (0.5 ng/ µl). The bioassay control dosing solution consisted of RNase-free water. Dosing solutions were prepared at room temperature.

Sample Collection

A sample of the test dosing solution was collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:5 dilution factor (30 µl test dosing solution to 120 µl QHS) on the day of preparation. Samples (~50 mg each) of Treatments 1 and 2 were collected into pre-weighed tubes during the process of diet aliquoting for the Springtail bioassay, as described in Table 71. The tubes containing diet were weighed again in order to obtain the exact sample weight. Samples were stored frozen if applicable (-80 °C freezer unit) until analysis.

Table 71. Springtail DvSSJ1 dsRNA Bioassay Test Dosing Solution Analyses

Treatment	Number of Samples	Analysis
1	5	Verify absence of DvSSJ1_210 dsRNA
2	5 Beginning	Homogeneity of DvSSJ1_210 dsRNA
	5 Middle	
	5 End	
	30 (3 each time point)	Frozen storage stability (-80 °C freezer unit)

Preparation of Sample Homogenates

Diet samples were removed from the freezer (if applicable) in preparation for extraction and analysis (three each from the beginning, middle, and end of diet aliquoting for homogeneity, three for each time point for frozen storage stability, and three for verification of the absence of DvSSJ1_210 dsRNA).

A volume of QHS (µl) equal to 10x sample weight (mg) was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were centrifuged again and the final supernatants (i.e., sample homogeneates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS; Treatment 1 samples were loaded to the plate undiluted. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the Treatment 2 dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and frozen storage stability), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solution

The concentration of DvSSJ1_210 dsRNA in test dosing solution was considered verified if the result fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed.

The acceptable range was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in the verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Verification of Absence of DvSSJ1_210 dsRNA in Treatment 1

Absence of DvSSJ1_210 dsRNA in Treatment 1 samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean MFI of lowest Standard Curve Point – 10%)

Verification of DvSSJ1_210 dsRNA Stability under Frozen Storage Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by time point (Days 8, 15, 23, 29, 37, and 44). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

E1.b. Springtail DvSSJ1 dsRNA Sensitive Insect Bioassay

The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 used in the Springtail bioassay was evaluated by conducting a 14-day bioassay using WCR, a species sensitive to DvSSJ1_210 dsRNA. The WCR bioassay was initiated after completion of the Springtail bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1).
- Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.057 ng DvSSJ1_210 dsRNA per mg *WCR* diet wet weight).

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 *WCR* individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 2 during diet aliquoting for the Springtail bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B on each day of diet preparation for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the Springtail bioassay, resulting in a 20% incorporation of the Springtail diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 2 from the Springtail bioassay, resulting in a 20% incorporation of the Springtail diet by dry weight of the WCR diet.

For each respective treatment, RNase-free water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparisons were made between WCR fed diet containing DvSSJ1_210 dsRNA (Treatment B) and the bioassay control diet (Treatment A) for the response variables.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

WCR fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was different from the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0\colon m_T - m_c = 0 \quad vs. \quad H_a\colon m_T - m_c \neq 0.$$

A significant difference was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups A and B; therefore, a two-sample *t*-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused larval growth inhibition. That is, to test if the weight of WCR larvae fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) was different from the weight of larvae fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0: w_T = w_C \qquad H_a: w_T \neq w_C$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatment A and B was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Analysis of WCR bioassay mortality and weight results can be found in Table 67 and Table 68, respectively.

E2. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to DvSSJ1 210bp dsRNA

Test Guideline: OECD Guidance Document No. 239

MATERIALS AND METHODS

Protocol

The procedures used in this study are based on an OECD Guidance Document No. 239 dated 15 July 2016 (OECD, 2016), procedures discussed in Protocol for the in vitro rearing of honey bee (*Apis mellifera* L.) workers (Schmehl *et al.*, 2016), and recent input from the U.S. EPA.

Test Substance and Water (for RNA work)

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under the stated storage condition.

The test substance was diluted by Pioneer Hi-Bred International, Inc., Johnston, Iowa, to create the DvSSJ1_210 dsRNA larval stock solution used in this study and was received on 24 May 2018.

Upon receipt at the contract research organization (CRO), the solution (No. 9443) was stored in a freezer (-80 °C) in the original containers.

Water (for RNA work)

The solution, water (for RNA work) was received on 24 May 2018 from Pioneer Hi-Bred International, Inc., Johnston, Iowa.

Upon receipt at the CRO, the solution (SMV No. 9447) was stored at room temperature in a dark, ventilated cabinet in the original container.

Test Organism

The honey bee larvae (*Apis mellifera*) used to initiate the definitive test were provided by Wood's Beekeeping Supply, Lincoln, Rhode Island. The source hives are identified as Italian hybrids as they contain a mixture of the original subspecies, *A. mellifera ligustica*, and unidentified subspecies which commonly co-exist in commercial bee hives. The queen from three or more hives was isolated on a single frame for one day to provide known-aged eggs and subsequent larvae. The frames were delivered to the CRO so that ≤24-hour old larvae (hatched within the previous 24 hours) were removed for testing. The hives from which the larvae were obtained were not previously exposed to any chemical treatments within four weeks of test initiation.

Culture and Test Vessels

The larval culture and test vessels were sterile, 48-well cell culture plates (1.6 mL/well; Corning) containing a plastic queen cup grafting cell (Mann Lake) in 32 wells during acclimation and in 18 wells during exposure. Each plate was labeled with the treatment or control, replicate, and study number. The perimeter wells within each plate not containing larvae were partially filled with deionized water to assist in maintaining the relative humidity at >90%.

The pupation plates were sterile, 24-well cell culture plates (3.4 mL/well; Corning) each containing two layers of sterilized dust-free Kimwipes.

Transfer of Larvae

Frames containing the isolated brood cells were removed from each hive, adult bees were removed from each frame, and the frames were then brought into the laboratory. Frames containing newly hatched larvae were placed in a grafting chamber maintained at approximately 26 to 32 °C. The larvae were removed from brood cells by placing a grafting tool under the larvae and carefully lifting it from its brood cell. To reduce the potential for contamination of pathogenic microorganisms, the transfer tool was sanitized with 70% ethyl alcohol after each transfer and blotted dry before use with the next larva. Care was taken to keep the larvae oriented to allow their spiracles access to air for respiration. Excess larvae were collected on day 1 to ensure enough were available for testing on day 3.

Acclimation Phase

The initial phase of the test involved a 2-day acclimation phase. Diets were prepared as described below. Larvae were fed 20 µL of untreated diet A on the day of transfer into the cell plates (day 1) and not fed on day 2. After the 2-day acclimation phase, dead or discolored larvae contained in a grafting cell were removed from the culture plates. Remaining healthy

larvae were randomly assigned to cell plates using computer generated random numbers until each treatment contained 12 larvae from three different hives for a total of 36 larvae per test group evenly divided between two plates (i.e., 18 larvae per plate) and placed in an environmental chamber.

Test Concentration Selection

Based on consultation with Pioneer Hi-Bred International, a target daily diet concentration of 0.0010 µg/a.i./larva/day was used to calculate a nominal cumulative dose of 0.0040 µg a.i./larva. This nominal cumulative dose and a control were selected for the definitive exposure.

Diet Preparation

The RNase-free water (No. 9447-1-A) used in the diet preparation was supplied by Pioneer Hi-Bred International. Three untreated diets were prepared prior to test initiation and were stored frozen until use. The diets were prepared as follows:

Component	Diet A[a]	Diet B[b]	Diet C[c]
RNase-free water (mL)	4.4	22	60
D-glucose[d] (g)	0.53	3.2	18
D-fructose[d] (g)	0.53	3.2	18
Yeast extract[de] (g)	0.090	0.65	4.0
Royal jelly[f] (g)	4.4	22	100

[a] Diet fed on day 1.

[b] Used for treated diet fed on exposure day 3.

[c] Used for treated diet fed on exposure days 4, 5, and 6.

[d] Supplier: Sigma Aldrich, Saint Louis, Missouri.

[e] Yeast extract is made from *Saccharomyces cerevisiae* species of yeast.

[f] Supplier: Stakich, Inc., Troy, Michigan.

NOTE: Royal jelly diet preparation ratios are based on modifications from Schmehl et al. (2016).

On feeding days, an aliquot of each diet was brought to test temperature by placing within the test incubator before being added to the plate wells. Thawed, untreated, and treated diet including unused diet was stored in a freezer when not in use. All diets were appropriately disposed of after final feeding.

Representative samples of the royal jelly diet were analyzed periodically for the presence of PCBs and toxic metals by Eurofins Lancaster Laboratories Environmental, Lancaster Pennsylvania using U.S. EPA standard methods (U.S. EPA, 1997). Additionally, a sample of royal jelly diet was analyzed for common pesticides and antibiotics by the U.S. Department of Agriculture (USDA), Gastonia, North Carolina. The diet was considered to be of acceptable quality since no analytes were measured at concentrations considered to be toxic to the test organisms (ASTM, 2007). The RNase-free water used to make diets was not part of the diet analysis for contaminates as it was expected to be free of pesticides due to the production process.

Preparation of Diets

Test Substance

A 0.00854 mg a.i./mL larval stock solution was prepared by Pioneer Hi-Bred International in RNase-free water for incorporation into royal jelly diet. An aliquot of stock solution was removed from storage and thawed at ambient temperature in order to dose diets used on days 3, 4, 5, and 6. Due to the different amount of diets offered to the test organisms and to provide a fixed daily dose, the diet concentrations were different on each exposure day as indicated in **Table 72**. A measured amount of stock solution was added to a batch of the appropriate diet.

Table 72. *Apis mellifera* Treated Diet Preparation Information:

Test Day	Dosing Stock Concentration (mg a.i./mL or µg a.i./µL)	Volume of Dosing Stock Solution Used (µL)	Total Weight of Diet Used (g)	Nominal Diet Concentration (µg a.i./g diet)	Total Amount Diet Dispensed per Well[a]		Nominal Daily Diet Dose (µg a.i./larva/day)	Nominal Cumulative Dose (µg a.i./larva)
					(µL)	(mg)		
3	0.00854	50.0	10	0.043	20	23	0.0010	0.0040
4	0.00854	33.3	10	0.028	30	35	0.0010	
5	0.00854	25.0	10	0.021	40	47	0.0010	
6	0.00854	20.0	10	0.017	50	59	0.0010	

[a] The weighted density is based on proportions of each diet type (B and C) within the total amount of diet dispensed per well (µL) as would be used in larval chronic testing. The weighted density is 1.1710 g/mL.

NOTE: Resulting diet concentrations and nominal dosages were calculated using the actual (unrounded) results and not the rounded values presented in this table.

All treated royal jelly diets appeared opaque and light yellow in color with no visible undissolved test substance after preparation. Untreated diet was used for the control.

Reference Toxicant

In order to assess the health of the hives used to provide the test population, an 8-day reference test was included in the test design and was conducted concurrently with the definitive exposure using larvae obtained from the same hives as those used to initiate the definitive exposure. Dimethoate was used as the reference toxicant and is known to be toxic to the honey bee larvae; therefore, the reference test was terminated at the end of the larval phase (day 8).

A 20 mg a.i./mL primary stock solution was prepared by bringing 2.0295 g of dimethoate (2.0254 g as active ingredient) to a volume of 100 mL with acetone (CAS No. 67-64-1). The resulting 20 mg a.i./mL reference toxicant solution was observed to be clear and colorless with no visible undissolved material following initial sonication of 20 seconds and mixing with a stir bar and magnetic stir plate for 10 minutes. A 12 mg a.i./mL solution was prepared by adding 6.0 mL of the 20 mg a.i./mL reference toxicant stock solution to a 10 mL volumetric flask and bringing it to volume with acetone (**Table 73**).

Table 73. *Apis mellifera* Larva Single treated diet Preparation Information:

Stock Concentration (mg a.i./mL)	Volume of Stock Solution Used (mL)	Total Weight of Diet (g)	Nominal Diet Concentration (µg a.i./g)	Total Amount Diet Dispensed per Well over Days 3, 4, 5, and 6[a]		Nominal Cumulative Dose (µg a.i./larva)
				(µL)	(mg)	
12	0.040	10	48	140	164	7.9

[a] The weighted density is based on proportions of each diet type (B and C) within the total 140 µL as would be used in larval chronic testing. The weighted density is 1.1710 g/mL.

NOTE: Resulting diet concentration was calculated using the actual unrounded results and not the rounded values presented in this table.

The resulting reference toxicant diet was observed to be opaque and yellow in color with no visible undissolved material following preparation.

Experimental Initiation

The test was initiated on day 3, when the appropriate treated or control diet B was added to the larval cell plates.

Feeding during Exposure Phase

On day 3, individual larvae in all plates were fed 20 µL of the appropriate diet B; on days 4, 5, and 6, respectively, all plates were fed 30, 40, and 50 µL of the appropriate diet C. Larvae that were observed to completely consume their diet on day 7 or 8 were transferred to the

appropriate, labeled pupation plates. On day 8, any larvae that did not consume the entire diet were considered dead.

Test Conditions

The larval exposure was conducted in the same incubator used for the acclimation phase, which was designed to maintain a temperature of 33 ± 2 °C with a relative humidity of ≥90%. The larvae, in their cell plates, were kept inside a plastic container in the incubator. The incubator maintained the test organisms in near darkness to approximate hive conditions. Organisms were exposed to laboratory lighting for approximately 30 minutes each day during observations and renewal of the diet.

Upon transfer to the pupal plates, the plates were maintained within an incubator at the same temperature but the relative humidity was designed to be maintained at 50 to 85%.

Temperature and relative humidity within a surrogate cell plate, placed in the incubator among the test plates, were monitored continuously using a HOBO data logger (Onset Computer Corporation, Model ZW-007).

Test Monitoring

The health of the larvae was observed and recorded daily. Death of a larva was defined by lack of movement. On days 7 and 8, all wells were observed for the presence of diet, and larvae that had not completely consumed their diet were considered dead. Survival of pupae was first checked on day 15 to avoid disturbing the fragile pre-pupal stage. Larvae that failed to develop into pupae by day 15 were classified as dead. Starting on day 15, the number of emerged adults each day was recorded. At the time of emergence, each adult bee was removed from the well plate and individually weighed on a Sartorius Model CPA225D balance to the nearest 0.0001 g, and discarded.

Experimental Termination

The test was terminated on day 22. At test termination, after health observations and remaining individual bee weights were recorded, any remaining organisms were frozen and discarded. Pupae that had not emerged as adults by day 22 were considered dead.

Analytical Measurements

Analytical measurements were conducted by Pioneer Hi-Bred International. Fifteen samples of test diet (five each collected from the beginning/middle/end of diet distribution) and five samples of control diet were collected on days 4, 5, and 6 of the exposure into tubes provided by Pioneer Hi-Bred International. In addition, all remaining aliquots of the larval stock solution

were returned to Pioneer Hi-Bred International. Results of these analyses were used to quantify the larval stock solution and confirm that treated diets were correctly prepared.

All samples were stored at -80 ± 10 °C prior to shipping. Samples were shipped on dry ice to Pioneer Hi-Bred International and were held frozen until analysis. Characterization of diets incorporated with DvSSJ1_210 dsRNA is presented in section **E2.a. Characterization of Larval and Adult Honey Bee Diets Incorporated with DvSSJ1 210bp dsRNA** below.

Endpoints and Statistical Analysis

The endpoints used for determination of significant effects by statistical evaluation are outlined below:

- Larval percent survival (number of live pupae on day 8 divided by the number of larvae exposed, 36, × 100)
- Pupal percent survival (number emerged adults on day 22 divided by the number alive on day 8 × 100)
- Day 22 percent emergence (number of emerged adults divided by the number exposed, 36, × 100)
- Honey bee weight at emergence

Determination of LOED and NOED Values

The treatment data was tested for normality and homogeneity of variance using the appropriate qualifying test. The Lowest-Observed-Effect Dose (LOED) is defined as the lowest dose that shows a statistically significant reduction and the No-Observed-Effect Dose (NOED) is the highest dose that shows no statistically significant reduction from the control. Basic guidance for this determination is presented in **Table 74** but the actual statistical analysis utilized was dependent on the data set.

Table 74. Summary of Apis mellifera DvSSJ1 dsRNA larva Bioassay Endpoints

Endpoint	Normal Distribution[a]	Equal Variance[b]	Statistical Test
Larval Survival **Pupal Survival** **Emergence**	**Not Applicable**	**Not Applicable**	**Monotonic Trend:** Cochran-Armitage's Step-Down Test **Non-Monotonic Trend:** Fisher's Exact Test with Bonferroni-Holm's Adjustment
Weight	**Yes**	**Yes**	**Monotonic Trend:** Williams' Multiple Comparison Test or Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunnett's Multiple Comparison Test
	No	**Yes or No**	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunn's Test or Wilcoxon's Test with Bonferroni-Holm's Adjustment
	Yes	**No**	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Tamhane-Dunnett Test or Dunnett's T3 Test

[a] Shapiro-Wilks' Test was used to assess normality of variance.

[b] Bartlett's Test was used to assess homoscedasticity.

All comparisons for determination of a NOED and LOED were made at ≥95% level of certainty ($p < 0.05$) and compared on a per replicate basis. A replicate was considered to be an individual larva/bee since they were reared in an individual cell. CETIS Version 1.8 was used to perform all statistical analysis. Results are reported in µg a.i./larva for cumulative dose (NOED and LOED values).

RESULTS

Larval Conditions

The daily minimum and maximum temperatures monitored in the cell plates ranged from 33 to 34 °C and relative humidity ranged from 89 to 97% during the larval phase (e.g., days 1 to 8,). Based on historical data, these parameters were considered acceptable for the survival and growth of the test organisms.

Pupal Conditions

The temperature ranged from 32 to 35 °C with a relative humidity range of 60 to 87% during the pupal phase (e.g., days 7 to 22). Based on historical data, these parameters were considered acceptable for the survival and growth of the test organisms.

Biological Results

DvSSJ1_210 Double-Stranded RNA Exposure

Table 76, Table 78, and Table 79, present the results of biological exposure. Table 80 provides a summary of percent survival, adult emergence, and adult weight at emergence. The larval survival (days 3 to 8) was 100 and 97% in the control and test treatments, respectively.

Fisher's Exact Test with Bonferroni-Holm's Adjustment determined no significant reduction in larval survival among honey bees exposed to the test compared to the control. Therefore, the 8-day larval survival NOED and LOED values for DvSSJ1_210 double-stranded RNA to honey bees were determined to be 0.0040 and >0.0040 µg a.i./larva, respectively.

The pupal survival (days 8 to 22) was 72 and 63% in the control and test treatments, respectively.

Fisher's Exact Test with Bonferroni-Holm's Adjustment determined no significant reduction in pupal survival among honey bees exposed to the 0.0040 µg a.i./larva treatment level compared to the control. Therefore, the 22-day pupal survival NOED and LOED values for DvSSJ1_210 double-stranded RNA to honey bees were determined to be 0.0040 and >0.0040 µg a.i./larva, respectively.

At test termination, the adult percent emergence (days 3 to 22) in the control and test treatments was 72 and 61% (15% relative difference), respectively.

Fisher's Exact Test with Bonferroni-Holm's Adjustment determined no significant reduction in adult percent emergence for honey bees exposed to the 0.0040 µg a.i./larva treatment level compared to the control. Therefore, the 22-day adult emergence NOED and LOED values for

DvSSJ1_210 double-stranded RNA to honey bees were determined to be 0.0040 and >0.0040 µg a.i./larva, respectively.

Mean live weight for adults at emergence is presented in Table 79. The mean adult weight at emergence in the control and test treatments was 0.1003 and 0.1060 g, respectively.

Statistical analysis (Equal Variance Two-Sample t-Test) determined no significant difference in weight between the control and the 0.0040 µg a.i./larva treatment. Therefore, the NOED and LOED values were determined to be 0.0040 and >0.0040 µg a.i./g larva, respectively.

Dimethoate Exposure (Reference Test)

Table 77 presents the results of biological exposure. The nominal cumulative dose rate of dimethoate maintained during the reference test was 7.9 µg a.i./larva equivalent to 48 µg a.i./g diet. Procedures used during the reference test were consistent with the procedures used in the definitive test. Mortality during the larval stage (days 3 to 8) was 97% for honey bee larvae exposed to 7.9 µg a.i./larva nominal cumulative dose. These results demonstrate that the larvae were sensitive to dimethoate in this exposure system.

Table 75. Apis mellifera Bioassay Acceptability Criteria

Acceptability Criteria	Study Results	Criterion Met (Yes/No)
Larval mortality from days 3 to 8 in the control, and solvent control, if present, should be ≤15% prior to pupation.	Larval mortality in the control was 0%.	Yes
Percent emergence in the control, and solvent control, if present, should be ≥70% at termination.	Emergence in the control was 72%.	Yes
Larval mortality in the reference toxicant treatment level (7.9 µg a.i. dimethoate/larva) should be ≥50% on day 8.	Larval mortality in the 7.9 µg a.i. dimethoate/larva treatment was 97%.	Yes

Conclusions

The 8-day larval, 22-day pupal, and 22-day percent emergence NOED and LOED values for DvSSJ1_210 double-stranded RNA to honey bees were all determined to be 0.0040 and >0.0040 µg a.i./larva, respectively. The live weight for adults at emergence NOED and LOED values for DvSSJ1_210 double-stranded RNA values were also determined to be 0.0040 and >0.0040 µg a.i./larva, respectively.

Table 81 summarizes the established endpoints for this study (LOED and NOED values) based on the nominal cumulative dose. Results of the toxic reference standard test (dimethoate) indicated that the test organisms were responsive to a toxicant of the appropriate dose in this study design.

Table 76. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to DvSSJ1_210 Double-Stranded RNA - Larval Survival and Mortality

Nominal Cumulative Dose (µg a.i./larva)	Percent Survival (Number of Surviving Larvae)					Day 8		
	Day 3	Day 4	Day 5	Day 6	Day 7	Percent Survival (Number of Surviving Larvae)	Percent Mortality (Number of Dead Larvae)	Abbott's Corrected Percent Mortality[a]
Control	100 (36)	100 (36)	100 (36)	100 (36)	100 (36)	100 (36)	0 (0)	NA[b]
0.0040	100 (36)	100 (36)	100 (36)	100 (36)	100 (36)	97 (35)	3 (1)	3

[a] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925). Statistical analysis was conducted using uncorrected values.

[b] NA = Not Applicable

Table 77. Dimethoate Reference Test for Apis mellifera Larval DvSSJ1 dsRNA Bioassay

Nominal Cumulative Dose (µg a.i./larva)	Cumulative Percent Survival (Number of Surviving Larvae)					Day 8	
	Day 3	Day 4	Day 5	Day 6	Day 7	Percent Survival (Number of Surviving Larvae)	Percent Mortality (Number of Dead Larvae)
7.9	100 (36)	94 (34)	64 (23)	3 (1)	3 (1)	3 (1)	97 (35)

NOTE: A total of 36 organisms per treatment or control at exposure initiation, day 3.

Table 78. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to DvSSJ1_210 Double-Stranded RNA - Pupal Survival and Adult Percent Emergence

Nominal Cumulative Dose (µg a.i./larva)	N[a]	Day 8 - 22			Day 3 - 22		
		Cumulative Percent Survival (Number of Surviving Pupae)	Cumulative Percent Mortality (Number of Dead Pupae)	Abbott's Corrected Percent Mortality[b]	Cumulative Percent Emergence[c] (Number of Emerged Adults)	Cumulative Percent Mortality[c] (Number of Dead Organisms)	Abbott's Corrected Percent Mortality[b]
Control	36	72 (26)	28 (10)	NA[d]	72 (26)	28 (10)	NA
0.0040	35	63 (22)	37 (13)	13	61 (22)	39 (14)	15

[a] N = the number of larvae transferred

[b] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925). Statistical analysis was conducted using uncorrected values.

[c] Based on 36 larvae at initiation

[d] NA = Not Applicable

Table 79. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to DvSSJ1_210 Double-Stranded RNA - Adult Weight at Emergence

Nominal Cumulative Dose (µg a.i./larva)	N[a]	Mean Adult Weight at Emergence[b] (g)
Control	26	0.1003 (0.0130)
0.0040	22	0.1060 (0.0165)

[a] N = the number of adults weighed

[b] Standard deviations are presented in parentheses.

Table 80. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to DvSSJ1_210 Double-Stranded RNA - Summary of the Percent Survival, Adult Emergence, and Adult Weight at Emergence

Nominal Cumulative Dose (µg a.i./larva)	8-Day Larval Survival[a] (%)	22-Day Pupal Survival[b] (%)	22-Day Percent Emergence[c] (%)	Adult Weight at Emergence (g)
Control	100	72	72	0.1003
0.0040	97	63	61	0.1060

[a] Based on observations from days 3 to 8

[b] Based on observations from days 8 to 22

[c] Based on observations from days 3 to 22

Table 81. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to DvSSJ1_210 Double-Stranded RNA - Endpoint Summary

Endpoint	Based on Nominal Cumulative Dose (µg a.i./larva)	
	NOED[a]	LOED[b]
3 - 8-Day Larval Survival	0.0040	>0.0040
8 - 22-Day Pupal Survival	0.0040	>0.0040
3 - 22-Day Adult Emergence	0.0040	>0.0040
Adult Weight at Emergence	0.0040	>0.0040

[a] NOED = No-Observed-Effect Dose

[b] LOED = Lowest-Observed-Effect Dose

E2.a. Characterization of Larval and Adult Honey Bee Diets Incorporated with DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Experimental Design

DvSSJ1_210 dsRNA test substance was used undiluted (2.1 mg/ml) for preparing adult honey bee diets. For the larval honey bee bioassay, DvSSJ1_210 dsRNA test substance was diluted in RNase-free water to a concentration of 8.54 ng/µl to create a larval stock solution. Aliquots of the test substance and larval stock solution were shipped to the CRO and used to prepare adult and larval honey bee diets, respectively. In addition, extra aliquots of larval stock solution were

shipped to The CRO and then returned once the honey bee bioassays were complete. These aliquots were used in the sensitive insect bioassay to confirm the biological activity of DvSSJ1_210 dsRNA and for concentration verification. Prepared diet samples from the honey bee bioassays were shipped to Pioneer Hi-Bred International, Inc. (referred to as Pioneer) for characterization.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in larval stock solution, homogeneity of larval test diets, concentration of DvSSJ1_210 dsRNA in adult test diets, and absence of DvSSJ1_210 dsRNA in adult and larval control diets. Homogeneity verification of the adult honey bee diet was not applicable because the diet was a solution. A sensitive insect bioassay with *Diabrotica virgifera virgifera* (section E2.b. *Apis mellifera* Larval Sensitive Insect Bioassay) was used to demonstrate the biological activity of DvSSJ1_210 dsRNA in the stored larval stock solution.

Control of bias during characterization was achieved through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. In addition, the larval stock solution and standard curve were prepared in bulk, minimizing day to day bias. Bias in the *WCR* bioassay was controlled through the randomization of treatments within blocks.

Preparation and Shipping of DvSSJ1_210 dsRNA Solutions

DvSSJ1_210 dsRNA test substance (2.1 mg/ml) was removed from frozen storage, allowed to thaw at ambient temperature, and aliquoted for use in the adult honey bee bioassay. A portion of the test substance was diluted in RNase-free water to a concentration of 8.54 ng/µl to create a larval stock solution and then aliquoted for use in the larval honey bee bioassay. All aliquots were stored frozen (-80 °C freezer unit).

Aliquots of the DvSSJ1_210 dsRNA test substance and larval stock solution were shipped to the CRO on dry ice for use in preparing adult and larval honey bee diets, respectively. Additional aliquots of the larval stock solution were also included in the shipment to the CRO; those aliquots were returned to Pioneer on dry ice after completion of the honey bee bioassays and used to verify the concentration in the larval stock solution and to prepare test diet for the sensitive insect bioassay.

Diet Sample Collection

Diets were prepared at the CRO for use in larval and adult honey bee bioassays. During the process of diet distribution, samples were collected as follows:

On days 4, 5 and 6 of the larval honey bee bioassay, 15 samples of test diet (50 µl each) were collected, five each from the beginning, middle, and end of diet distribution for homogeneity

verification. In addition, 5 samples of control diet (50 µl each) were collected on each of those days to verify the absence of DvSSJ1_210 dsRNA.

On days 2, 3, 7, and 9 of the adult honey bee bioassay, 5 samples of test diet (200 µl each) and 5 samples of control diet (200 µl each) were collected for verification of the concentration or absence of DvSSJ1_210 dsRNA in adult test and control diets, respectively.

Characterization of Larval Stock Solution and Larval and Adult Honey Bee Diets

Larval Stock Solution and Adult Honey Bee Diets

The larval stock solution was analyzed on the day of preparation by diluting in QuantiGene Homogenizing Solution (QHS) to fall within the range of the standard curve. The larval stock solution was analyzed again using a returned aliquot following final use in the larval honey bee bioassay. Aliquots of the DvSSJ1_210 dsRNA adult honey bee diets were prepared for analysis by diluting in QHS to fall within the range of the standard curve. Adult honey bee control diets were analyzed without dilution. Three samples were analyzed for each time point, for each diet.

Preparation of Sample Homogenates for Larval Diets

Larval honey bee diet samples were removed from the freezer in preparation for extraction and analysis (nine test diet samples per time point for homogeneity three each from the beginning, middle, and end of diet distribution and three control diet samples per time point for verification of the absence of DvSSJ1_210 dsRNA).

QHS (500 µl) was added to each tube containing diet; samples were then vortexed continuously for four minutes, and centrifuged. The supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Control diet samples were loaded to the plate undiluted. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation

with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the larval stock solution and adult honey bee diets was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Larval Honey Bee Stock Solution and Adult Honey Bee Diet

The concentration of DvSSJ1_210 dsRNA in the larval stock solution and adult honey bee diet was considered verified if the results fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Larval Honey Bee Diets

The mean concentration $\overline{x}$ of the test substance, expressed in MFI, was determined across all larval honey bee diet samples analyzed for a given diet preparation day.

The acceptable range for each diet preparation day was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

For the Day 5 homogeneity samples, two of the nine samples fell out of range during initial analysis; therefore, the remaining six samples for Day 5 were prepared for analysis as described in above and all 15 samples were analyzed on the same plate. On re-analysis, the same two samples were still out of range; one sample was excluded from calculations due to a documented sample handling error.

For Days 4 and 6, all samples were within the acceptable range; therefore, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity for Days 4 and 6.

For Day 5, 1 out of 14 samples fell out of range; therefore, the estimated probability of a sample falling outside of the range was 0.0714. A 95% exact (Clopper and Pearson, 1934) confidence interval for the estimated probability of samples falling out of range was calculated as 0.0018 - 0.3387.

Verification of Absence of DvSSJ1_210 dsRNA in Larval and Adult Honey Bee Control Diets

Absence of DvSSJ1_210 dsRNA in larval and adult honey bee control diet samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean MFI of Lowest Standard Curve Point – 10%)

Demonstration of DvSSJ1_210 dsRNA Activity in Larval Stock Solution

Aliquots of the stored DvSSJ1_210 dsRNA larval stock solution were returned to Pioneer by the CRO on dry ice after completion of the larval and adult honey bee bioassays. The returned aliquots were used to prepare Treatment B in a sensitive insect bioassay using *WCR* larvae to demonstrate the biological activity of DvSSJ1_210 dsRNA in the honey bee diets. Details regarding the sensitive insect bioassay are provided in section E2.b. *Apis mellifera* Larval Sensitive Insect Bioassay.

Results and Discussion

DvSSJ1_210 dsRNA test substance and larval stock solution were prepared by Pioneer and used by the CRO to prepare adult and larval honey bee diets, respectively. Samples of the prepared diets were shipped to Pioneer for characterization by QuantiGene analysis.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in larval stock solution and adult test diets, and the absence of DvSSJ1_210 dsRNA in control diets for both honey bee bioassays (Table 82 and Table 83). The homogeneity of DvSSJ1_210 dsRNA in larval test diets was verified for Day 4 and Day 6 samples (Table 82); all homogeneity samples analyzed for Days 4 and 6 (9 per day) were within the acceptable range. One of fourteen Day 5 samples analyzed exceeded the acceptable range of 30% of the mean; however, this single sample was within 40% of the mean. In addition, the upper confidence limit for the estimated probability of a sample falling outside of the acceptable range (33.87%) was determined to be within acceptable parameters for the Day 5 diet.

Therefore, given that the other 13 samples analyzed were within range, homogeneity of DvSSJ1_210 dsRNA in the Day 5 diet was considered verified.

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 84. The WCR bioassay met the acceptability criterion (Section E2.b. *Apis mellifera* Larval Sensitive Insect Bioassay). The stored larval stock solution used in the larval honey bee bioassay was diluted from the same vial of test substance used for the adult honey bee bioassay. Aliquots of this solution were returned to Pioneer and the biological activity of the DvSSJ1_210 dsRNA in the larval stock solution was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B) when compared to the bioassay control diet (Treatment A).

Conclusion

DvSSJ1_210 dsRNA test substance was provided to the CRO for use in larval and adult honey bee diets and the diets incorporating the test substance were characterized.

Table 82. Verification of DvSSJ1_210 dsRNA Concentration in Larval Stock Solution, Homogeneity in Larval Honey Bee Test Diet, and Absence in Control Diet

Analysis	Time Point	Result
Larval Stock Solution Concentration Verification	Day of Preparation	120% of expected[a]
	After Final Use	114% of expected[a]
Homogeneity in Larval Honey Bee Test Diet	Bioassay Day 4	Verified[b]
	Bioassay Day 5	Verified[b]
	Bioassay Day 6	Verified[b]
Absence in Control Diet	Bioassay Day 4	Verified[c]
	Bioassay Day 5	Verified[c]
	Bioassay Day 6	Verified[c]

[a] Result is the average of three results for each time point; concentrations within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified for a given time point if the value of each sample was within 70-130% of the mean value of all samples analyzed for that time point. On Day 5, one of 14 samples exceeded the acceptable range; however, the single sample was within 40% of the mean.

[c] Absence of DvSSJ1_210 dsRNA in the control diet was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

Table 83. Verification of DvSSJ1_210 dsRNA Concentration in Adult Honey Bee Test Diet and Absence in Control Diet

Analysis	Time Point	Result
Concentration Verification in Adult Honey Bee Test Diet	Bioassay Day 2	84% of expected[a]
	Bioassay Day 3	110% of expected[a]
	Bioassay Day 7	118% of expected[a]
	Bioassay Day 9	121% of expected[a]
Absence in Control Diet	Bioassay Day 2	Verified[b]
	Bioassay Day 3	Verified[b]
	Bioassay Day 7	Verified[b]
	Bioassay Day 9	Verified[b]

[a] Result is the average of three results for each time point; concentrations within 70-130% of the expected value were considered verified.

[b] Absence of DvSSJ1_210 dsRNA in the control diet was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

Table 84. Summary of Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	30	6.67	28	1.55 ± 0.670	0.3 - 2.7
B	Test Diet	1	29[a]	89.7	3	0.267 ± 0.153	0.1 - 0.4

Note: Treatment B was prepared from the same stored larval stock solution used to prepare diets for the larval honey bee bioassay; the larval stock solution was prepared from the same vial of test substance used to create adult honey bee diets. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

E2.b. *Apis mellifera* Larval DvSSJ1 dsRNA Sensitive Insect Bioassay

The biological activity of DvSSJ1_210 dsRNA in the stored larval stock solution used to create larval honey bee diets and prepared from the same vial of test substance used to create adult honey bee diets was evaluated by conducting a 14-day bioassay using *Diabrotica virgifera virgifera* (*WCR*), a species sensitive to DvSSJ1_210 dsRNA. The *WCR* bioassay was initiated after return of the stored larval stock solution by The CRO following final use in the honey bee bioassays.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the *WCR* bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (prepared with RNase-free water)
- Treatment B: Test Diet (prepared with stored DvSSJ1_210 dsRNA larval stock solution and targeting 1 ng DvSSJ1_210 dsRNA per mg *WCR* diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 *WCR* individuals.

Diet Preparation

An aliquot of the stored DvSSJ1_210 dsRNA larval stock solution returned from The CRO was removed from frozen storage (-80 °C freezer unit), thawed, and diluted in RNase-free water to the appropriate concentration (1.4 ng/µl) to create a bulk dosing solution for use in Treatment B; the dosing solution was stored in a refrigerator when not in use. Treatments A and B for the *WCR* bioassay were prepared as follows:

- For Treatment A, RNase-free water was mixed with artificial diet for the *WCR* bioassay at a 2.51:1 ratio (*i.e.*, 2.51 ml water to 1 g carrier).
- For Treatment B, DvSSJ1_210 dsRNA dosing solution was mixed with artificial diet for the *WCR* bioassay at a 2.51:1 ratio (*i.e.*, 2.51 ml dosing solution to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated a bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 78 Weight data were summarized in Table 79.

E3. 14-Day Oral Toxicity Test with the Adult Honey Bee (*Apis mellifera*) Exposed to DvSSj1 210bp dsRNA

MATERIALS AND METHODS

Protocol

The methods described in this protocol are based on the testing requirements of the OECD 245 Guideline for honey bee (*Apis mellifera* L.), chronic oral toxicity test (10-day feeding), OECD, 2017, and recent input from the U.S. EPA. The exposure was extended to 14 days at Pioneer's request.

Test Substance and Water (for RNA work)

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under the stated storage condition. The test substance was received on 24 May 2018 from Pioneer Hi-Bred International, Inc., Johnston, Iowa.

Upon receipt at The CRO, the test solution (SMV No. 9444) was stored in a freezer (-80 °C) in the original containers.

Determination of stability and characterization, verification of the solution identity, maintenance of records on the solution, and archival of a sample of the solution are the responsibility of Pioneer Hi-Bred International.

Water (for RNA work)

The solution, water (for RNA work), was received on 24 May 2018 from Pioneer Hi-Bred International, Inc., Johnston, Iowa. The following information was provided:

Upon receipt at the Contract Research Organization (CRO), the solution (No. 9447) was stored at room temperature in a dark, ventilated cabinet in the original container.

Test Organism

The honey bees (*Apis mellifera*) used during this study were ≤2-day old emerged adult bees removed from isolated brood frames containing known aged bees from hives provided by Wood's Beekeeping Supply, Lincoln, Rhode Island. The source hives are identified as Italian hybrids as they contain a mixture of the original subspecies, *A. mellifera ligustica*, and unidentified subspecies, which commonly co-exist in commercial bee hives. The honey bees were from hives that had not previously been exposed to chemical application for at least one month prior to use in testing. Brood frames containing capped brood with no emerged

adult bees present were received one day prior to acclimation. Bees that emerged from the brood frames were less than one day old from emergence when manually selected and impartially placed in acclimation vessels. The honey bees were transferred into the test vessels by inserting a bee into the test vessel one at a time until ten bees were added. Vessels containing organisms were then placed into an environmental chamber and acclimated to test conditions until exposure to the dosed diets on the following day. During the 1-day acclimation period, the test organisms were supplied with 2.0 mL of 50% untreated sucrose solution diet, prepared by dissolving an equal portion of food-grade sucrose in RNase-free water (w/w). The sucrose solution diet was provided in a syringe inserted through the top of the vessel.

Representative samples of 50% sucrose solution (comprised of water and organic sucrose) were analyzed periodically for the presence of PCBs and selected toxic metals by Eurofins Lancaster Laboratories Environmental, Lancaster, Pennsylvania (U.S. EPA, 1997). Representative samples of 50% sucrose solution were analyzed for the presence of pesticides by the U.S. Department of Agriculture, Gastonia, North Carolina. Each analyte was considered to be of acceptable quality since no analytes were measured at concentrations considered to be toxic to the test organisms (ASTM, 2007). The RNase-free water used to make diets was not part of the diet analysis for contaminates as it was expected to be free of pesticides due to the production process.

Test Vessels

Test vessels were 120-mL glass jars with screw top lids. Ventilation holes and a larger hole for insertion of the feeding syringe were drilled in the lid.

Replication

Three replicate vessels (1, 2, and 3) were established for the treatment level and control group. Each replicate contained 10 honey bees (30 honey bees per treatment level and control). Each replicate vessel was randomly assigned to a treatment or control group using a random number generator prior to the start of the exposure. Each jar was labeled to identify the dose level or control, replicate identification, and study number.

Test Conditions

The 14-day exposure was conducted in an environmental chamber designed to maintain a temperature of 33 ± 2 °C and a relative humidity ranging from 50 to 70%. The environmental chamber maintained the test organisms in near darkness to approximate hive conditions. Honey bees were exposed to laboratory lighting for approximately 30 minutes each day during observations and renewal of sucrose diets.

Test Concentration Selection

Based on consultation with Pioneer Hi-Bred International, a target daily diet concentration of 0.81 mg a.i./kg diet was used to calculate a nominal dose of 0.020 µg a.i./bee/day. This nominal dose and a control were chosen for the limit test.

Test Substance

Test substance (2.1 µg a.i./µL) was provided by Pioneer Hi-Bred International for incorporation into 50% sucrose solution diet made with RNase-free water. On a daily basis, an aliquot of test substance was removed from storage and thawed at room temperature in order to dose diets used on days 0 through 13. A measured amount of test substance was added to a batch of the appropriate diet.

Diet solutions were mixed with a stir bar and stir plate for at least 10 minutes. All resulting diet solution were observed each day to be amber in color due to the sucrose in solution.

The control vessels received only fresh, untreated sucrose solution.

Reference Toxicant

In order to assess the sensitivity of the bees used in testing, a reference test was included in the test design and was conducted concurrently with the limit test using honey bees obtained from the same hives as those used to initiate the limit test. Dimethoate, a known honey bee toxicant, was used in the reference test.

A 20 mg a.i./mL primary stock solution was prepared by bringing 2.0295 g of dimethoate (2.0254 g as active ingredient) to a volume of 100 mL with acetone (CAS No. 67-64-1). The resulting 20 mg a.i./mL reference toxicant solution was observed to be clear and colorless with no visible undissolved material following initial sonication of 20 seconds and mixing with a stir bar and magnetic stir plate for 10 minutes. A 0.062 mg a.i./mL solution was prepared by adding 0.31 mL of the 20 mg a.i./mL reference toxicant stock solution to a 100-mL volumetric flask and bringing it to volume with acetone. A single treated diet was prepared using the preparation scheme in **Table 85**.

Table 85 Apis mellifera Adult Single Treated Diet Information

Primary Stock Concentration (mg a.i./mL)	Volume of Primary Stock Solution Used (mL)	Volume Diluted to with Acetone (mL)	Secondary Stock Concentration (mg a.i./mL)	Volume of Stock Used (mL)	Volume Diluted to with 50% Sucrose Diet (mL)	Final Diet Concentration	
						Nominal Diet Concentration (mg a.i./kg)[a]	Nominal Dose (µg a.i./bee/day)[b]
20	0.31	100	0.062	0.50	25	1.0	0.025

[a] 0.00124 mg a.i.//mL (1.0 mg a.i./kg) based on a 50% Sucrose solution density of 1232.02 mg/mL (USDA, 1981).

[b] Theoretical value based on an estimated consumption rate of 200 µL (246 mg) per 10 bees per day.

NOTE: Results were calculated using the actual unrounded results and not the rounded values presented in this table.

The diet solution was mixed using a stir bar and stir plate for at least 20 minutes and for the diet solution prepared on day 3, hand shaking with inversions. The resulting reference toxicant diet was observed to be clear and amber in color with no visible undissolved material following preparation. The final nominal diet concentration was 1.0 mg a.i./kg of diet solution, equivalent to a dose rate of 0.025 µg a.i./bee/day. This diet solution was prepared on test days 0, 3, 6, 9, and 12 and used for the test days as referenced in Table 86.

Table 86. *Apis mellifera* Diet Solution Preparation Day and Days of Use in Testing.

Day of Secondary Diet Preparation	Days of Use in Testing
0	0 - 2
3	3 - 5
6	6 - 8
9	9 - 11
12	12 - 13

Experimental Initiation

The theoretical feeding rate of 200 µL per 10 bees per day was assumed based on past consumption rates and published data (Decourtye *et al.*, 2005). Complete consumption of 200 µL per vessel per day along with analytical recoveries that closely approximated nominal values would provide the expected nominal dose. Sucrose solution diet was administered to each test vessel using a 3.0-mL plastic syringe with the tip removed, containing approximately 2.0 mL of the appropriate diet. Syringes were filled with the appropriate diet on a daily basis.

The actual amount of diet consumed per vessel was verified throughout the test by weighing each syringe before and after each daily feeding to the nearest 0.0001 g using a Sartorius (Secura 225D-1S) analytical balance. The following table details how diet concentrations were used to derive each dose rate:

Table 87. *Apis mellifera* Adult Final Diet DvSSJ1 dsRNA Concentration and Nominal Dose Rate

Final Diet Concentration (mg a.i./kg)	Theoretical Amount Consumed per Vessel (µL/day)	Number of Bees per Vessel	Nominal Dose Rate per Bee (µg a.i./bee/day)[a]
0.81	200	10	0.020

[a] 50% Sucrose solution density = 1232.02 mg/mL (USDA, 1981).

NOTE: Results were calculated using the actual unrounded results and not the rounded values presented in this table.

Each feeding syringe and vessel was labeled to identify the concentration, replicate, and study number. Bees were allowed to feed on the diets *ad libitum* each day during the 14-day exposure.

Test Monitoring

Observations of the honey bees exposed to DvSSJ1_210 double-stranded RNA and the control were made daily. Mortality and any unusual behavior exhibited by the bees (e.g., apathy) were also recorded. Test organisms were considered dead if observed to be immobile on the bottom of the vessel, exhibiting no response to gentle prodding. At termination (day 14), all surviving bees were frozen and weighed on a Sartorius (Secura 225D-1S) analytical balance to the nearest 0.0001 g.

Feeding syringes containing diets were weighed daily before and after filling with freshly prepared diet solution. Additionally, three vessels were established, each with one syringe filled with untreated sucrose solution and without honey bees, and the syringe weight monitored daily for evaporative loss. Daily honey bee dose was then calculated from daily diet consumption, daily evaporative loss, and daily mortality to more accurately reflect the daily dose. Daily dose was calculated using the following formula:

$$Daily\ dose\ (\frac{\mu g}{bee}) = \left(\frac{mg\ food\ consumed}{bee} \times \frac{1\ mL}{1232.02\ mg\ *}\right) \times nominal\ diet\ concentration\ (\frac{\mu g}{mL})$$

* 50% Sucrose solution density = 1232.02 mg/mL (USDA, 1981).

NOTE: The amount of diet weight loss caused by evaporation is subtracted from the syringe weight difference over the feeding period before reported as mg food consumed.

Relative humidity within the environmental chamber and continuous temperature measurements were monitored continuously using a HOBO data logger (Onset Computer Corporation, Model ZW-007).

Analytical Measurements

Analytical measurements were conducted by Pioneer Hi-Bred International. Five samples of test and control diet were collected on days 2, 3, 7, and 9 of the exposure. In addition, all remaining aliquots of the test substance were returned to Pioneer Hi-Bred International. Results of these analyses were used to confirm that the treated diets were correctly prepared and t quantify actual exposure concentrations.

All samples were stored at -80 ± 10 °C prior to shipping. Samples were shipped on dry ice to Pioneer Hi-Bred International and were held frozen until analysis. Information on methods used during analysis are referenced in section E3.a. Characterization of Larval and Adult Honey Bee Diets Incorporated with DvSSJ1 210bp dsRNA.

Determination of the NOEC/NOEDD and LOEC/LOEDD Values

The endpoints for this study are expressed as nominal diet concentration (mg a.i./kg) and calculated mean daily dose (µg a.i./bee/day). The highest test concentration in diet and dietary dose that elicited no statistically significant reduction between the exposed organisms and the appropriate control (No-Observed-Effect Concentration, NOEC, and No-Observed-Effect Dietary Dose, NOEDD) were estimated. The lowest test concentration in diet and dietary dose that elicited a statistically significant reduction on organism performance (Lowest-Observed-Effect Concentration, LOEC, and Lowest-Observed-Effect Dietary Dose, LOEDD) were also estimated. The treatment data were tested for normality and homogeneity of variance using the appropriate qualifying test (Table 88). Basic guidance for this determination is as follows, but the actual analysis used is dependent on the data set:

Table 88. Apis mellifera Adult Bioassay Statistical Testing Summary

Endpoint	Normal Distribution[a]	Equal Variance[b]	Statistical Test
Percent Survival	Not Applicable	Not Applicable	**Monotonic Trend:** Cochran-Armitage's Step-Down Test **Non-Monotonic Trend:** Fisher's Exact Test with Bonferroni-Holm's Adjustment
Weight	Yes	Yes	**Monotonic Trend:** Williams' Multiple Comparison Test or Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunnett's Multiple Comparison Test
	No	Yes or No	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunn's Test or Wilcoxon's Test with Bonferroni-Holm's Adjustment
	Yes	No	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Tamhane-Dunnett's Test or Dunnett's T3 Test

[a] Shapiro-Wilks' Test was used to assess normality of variance.

[b] Variance Ratio F was used to assess homoscedasticity.

CETIS Version 1.8 was used to determine the NOEC/NOEDD and LOEC/LOEDD values.

RESULTS

Environmental Conditions

The temperature monitored within the environmental chamber ranged from 33 to 34°C and the relative humidity ranged from 59 to 68% during the study. Based on historical data, these conditions were within an acceptable range for honey bee survival.

Analytical Results

The diet characterization for each treatment was performed by Pioneer Hi-Bred International and the results are reported in section E2.a. Characterization of Larval and Adult Honey Bee Diets Incorporated with DvSSJ1 210bp dsRNA. As the analysis confirmed nominal concentrations, results are reported as nominal values.

Biological Results

DvSSJ1_210 double-stranded RNA Exposure

Based on feeding syringe weights recorded at the beginning and end of each exposure interval, the mean amount of diet consumed was 32 and 31 mg/bee/day for the control and test treatment levels, respectively, through 10 days of exposure. Based on the overall mean percent consumption rates, the control and test treatments both consumed 32 mg of diet per bee over the 14-day test. Food consumption by treatment is presented in Table 90. These data indicate that the diet consumption in the test treatment was the same as the control consumption rate.

Table 91 presents the calculated mean daily dose rates tested, corresponding daily percent survival, and day 10 and day 14 percent mortality observed during the 14-day oral exposure.

Following 14 days of exposure, a percent mortality of 10% was observed among honey bees exposed to both the test and the control treatments. Fisher's Exact Test with Bonferroni Holm's Adjustment indicated no significant difference in mortality in the test treatment relative to the control. The 10- and 14-day NOEC and NOEDD value for mortality were determined to be 0.81 mg a.i./kg and 0.026 µg a.i./bee/day, respectively (Table 94). The 10- and 14-day LOEC and LOEDD value for mortality was determined to be >0.81 mg a.i./kg and >0.026 µg a.i./bee/day, respectively (Table 94).

Table 93 presents the calculated mean daily dose rates tested and corresponding mean body weights measured following the 14-day oral exposure. Mean body weight of 0.1007 g was observed among honey bees exposed to the test treatment. Mean weight of 0.1047 g was observed among the control honey bees. The 14-day NOEC and NOEDD values for weight were

determined to be 0.81 mg a.i./kg diet and 0.026 µg a.i./bee/day, respectively (Table 94). The 14-day LOEC and LOEDD values for weight were determined to be >0.81 mg a.i./kg diet and >0.026 µg a.i./bee/day, respectively (Table 94).

Dimethoate Exposure (Reference Test)

Table 92 presents the results of the biological exposure. The nominal dose of dimethoate maintained during the reference test was 0.025 µg a.i./bee/day. Procedures used during the reference test were consistent with the procedures used in the limit test. The percent mortality on day 10 was 100% in the 0.025 µg a.i./bee/day nominal dose. The results demonstrate that the honey bees were sensitive to dimethoate in this exposure system and the mortality was >50% at this dose as expected in the draft guideline.

Acceptability Criteria

Required acceptance criteria are presented in **Table 89** .

Table 89. Apis mellifera Adult Bioassay Acceptability Criteria

Acceptability Criteria	Study Results	Criterion Met
Mean mortality in the control must not equal or exceed 15% at termination.	Cumulative percent mortality in the control was 10% at test termination.	Yes
Mean mortality in the reference test must be ≥50% after 10 days of exposure.	Percent mortality of 100% was observed during the dimethoate reference test.	Yes

Conclusions

The results from the diet analyses indicated the appropriate exposure concentrations were maintained during the study (section E2.a. Characterization of Larval and Adult Honey Bee Diets Incorporated with DvSSJ1 210bp dsRNA). The 10- and 14-day percent survival NOEDD and LOEDD values for DvSSJ1_210 double-stranded RNA to honey bees were determined to be 0.026 and >0.026 µg a.i./bee/day, respectively. The 14-day honey bee weight NOEDD and LOEDD values for DvSSJ1_210 double-stranded RNA to honey bees were determined to be 0.026 and >0.026 µg a.i./bee/day, respectively.

Table 94 summarizes the established endpoints for this study (NOEC/NOEDD and LOEC/LOEDD values) based on nominal diet concentrations and calculated mean daily dose. Results of the toxic reference standard test (dimethoate) indicated that the test organisms were responsive to a toxicant in this study design.

Table 90. 14-Day Oral Exposure of Honey Bees (*Apis mellifera*) to DvSSJ1_210 double-stranded RNA - Food Consumption, Calculated Mean Daily Dose, and Accumulated Dose

10-day

Nominal Dose (µg a.i./bee/day)	Nominal Diet Concentration (mg a.i./kg)	Overall Mean Daily Consumption of Food Solution (mg/bee/day)[a]	Calculated Mean Daily Dose (µg a.i./bee/day)[b]	Mean Accumulated Dose (µg a.i./bee)
Control	NA[c]	32	NA	NA
0.020	0.81	31	0.026	0.26

14-day

Nominal Dose (µg a.i./bee/day)	Nominal Diet Concentration (mg a.i./kg)	Overall Mean Daily Consumption of Food Solution (mg/bee/day)[a]	Calculated Mean Daily Dose (µg a.i./bee/day)[b]	Mean Accumulated Dose (µg a.i./bee)
Control	NA[c]	32	NA	NA
0.020	0.81	32	0.026	0.36

[a] Daily honey bee diet consumption was corrected for average, daily evaporative loss (overall mean daily evaporation was 56 mg).

[b] Calculated mean daily dose (µg a.i./bee/day) = mean diet consumed (mg/bee) × nominal concentration (mg a.i./kg).

[c] NA = Not Applicable

NOTE: Values were calculated from the raw data and not the rounded values presented in this table.

Table 91.14-Day Oral Exposure of Honey Bees (*Apis mellifera*) to DvSSJ1_210 double-stranded RNA - Survival and Mortality
10-day

Nominal Daily Dose (µg a.i./bee/day)	Replicate	Percent Survival										Day 10	
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Cumulative Percent Mortality Day 10	Abbott's Corrected Percent Mortality[a]
Control	1	100	100	100	100	100	100	100	100	100	100	0	
	2	100	100	100	100	100	100	100	90	90	80	20	
	3	100	100	100	100	100	100	100	90	90	90	10	
	Mean (SD[b])	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**93 (6)**	**93 (6)**	**90 (10)**	**10 (10)**	**NA[c]**
0.020	1	100	100	100	100	100	100	100	100	100	100	0	
	2	100	100	100	100	100	100	100	100	100	100	0	
	3	100	100	100	100	100	100	100	100	100	100	0	
	Mean (SD)	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**0 (0)**	**-11**

14-day

Nominal Daily Dose (µg a.i./bee/day)	Replicate	Percent Survival					Day 14	
		Day 10	Day 11	Day 12	Day 13	Day 14	Cumulative Percent Mortality Day 14	Abbott's Corrected Percent Mortality[a]
Control	1	100	100	100	100	100	0	
	2	80	80	80	80	80	20	
	3	90	90	90	90	90	10	
	Mean (SD)	**90 (10)**	**90 (10)**	**90 (10)**	**90 (10)**	**90 (10)**	**10 (10)**	**NA**
0.020	1	100	100	100	100	90	10	
	2	100	100	100	100	80	20	
	3	100	100	100	100	100	0	
	Mean (SD)	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**90 (10)**	**10 (10)**	**0**

[a] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925). Statistical analysis was conducted using uncorrected value.

[b] SD = Standard Deviation

[c] NA = Not Applicable

NOTE: A total of 30 organisms per dose or control were exposed at test initiation.

Table 92. 14-Day Oral Exposure of Honey Bees (*Apis mellifera*) to the Reference Toxicant, Dimethoate - Mortality
10-day

Nominal Dose (µg a.i./bee/day)[a]	Cumulative Percent Survival (Number of Dead Larvae)									Day 10
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Percent Mortality (Number of Dead Larvae)
0.025	100 (0)	100 (0)	97 (1)	93 (2)	57 (13)	23 (23)	10 (27)	0 (30)	0 (30)	100 (30)

[a] Equivalent to 1200 µg a.i./L of diet, or 1.0 mg a.i./kg of diet.

[b] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925).

NOTE: A total of 30 organisms per dose were exposed at test initiation.

Table 93. 14-Day Oral Exposure of Honey Bees (*Apis mellifera*) to DvSSJ1_210 double-stranded RNA - Weight
14-day

Calculated Mean Daily Dose (µg a.i./bee/day)	N	Mean Weight, g (SD[a])
Control	27	0.1047 (0.0026)
0.026	27	0.1007 (0.0037)

[a] SD = Standard Deviation

Table 94. 14-Day Oral Exposure of Honey Bees (*Apis mellifera*) to DvSSJ1_210 double-stranded RNA - Endpoint Summary

Endpoint	Nominal Diet (mg a.i./kg)	Calculated Mean Daily Dose (µg a.i./bee/day)
	10-day Percent Survival	
NOEC/NOEDD	0.81	0.026
LOEC/LOEDD	>0.81	>0.026
	14-day Percent Survival	
NOEC/NOEDD	0.81	0.026
LOEC/LOEDD	>0.81	>0.026
	14-day Live Adult Weight	
NOEC/NOEDD	0.81	0.026
LOEC/LOEDD	>0.81	>0.026

E3.a. Characterization of Larval and Adult Honey Bee Diets Incorporated with DvSSJ1 210bp dsRNA

This information is presented in section E2.a. Characterization of Larval and Adult Honey Bee Diets Incorporated with DvSSJ1 210bp dsRNA.

E4. Evaluation of the Survival of Parasitic Hymenoptera Fed Artificial Diets Containing DvSSJ1 210 bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of 30% sucrose prepared weight by volume (w/v) with RNase-free water.

Test System

The test system was Pediobius foveolatus (Hymenoptera: Eulophidae; Parasitic Hymenoptera). Parasitic Hymenoptera was selected as a representative non-target organism to characterize DvSSJ1_210 dsRNA. Parasitic Hymenoptera pupae were obtained from State of New Jersey Department of Agriculture (Trenton, NJ, USA) and identity was recorded by study personnel.

Experimental Design

Parasitic Hymenoptera adults were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing sucrose in RNase-free water)
- Treatment 2: Test Diet (targeting 1 µg DvSSJ1_210 dsRNA per ml sucrose diet)
- Treatment 3: Positive Control Diet (targeting 8000 µg boric acid per ml sucrose diet)

Treatments were arranged in a generalized randomized block design with a total of 3 blocks. Each block consisted of 1-oz plastic cups in a 30-well tray and contained 10 replicates from each treatment. Each treatment was provided to a target of 30 Parasitic Hymenoptera individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Adults were refed daily. After 14 days, the bioassay was complete and mortality was assessed. Mortality was statistically compared between Parasitic Hymenoptera provided Treatment 1 and those provided Treatment 2.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.

- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

QuantiGene analysis was used to verify the dose and stability under frozen storage conditions of DvSSJ1_210 dsRNA in Treatment 2 and the absence of DvSSJ1_210 dsRNA in Treatment 1. Sensitive insect bioassays were used to demonstrate the biological activity of DvSSJ1_210 dsRNA in Treatment 2 of the Parasitic Hymenoptera bioassay.

Bias in the Parasitic Hymenoptera bioassay and sensitive insect bioassays was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

The bioassay control diet (Treatment 1) and test diet (Treatment 2) were each prepared in bulk as described in section E4.a. Parasitic Hymenoptera Bioassay Analytical Phase. Treatment 3 was prepared in bulk by combining a boric acid stock solution and 50% sucrose (w/v), each prepared with RNase-free water, to achieve a nominal concentration of 8000 µg boric acid per ml and 30% sucrose. Each diet was aliquoted into individual microcentrifuge tubes (200 µl per tube) and a square of autoclaved tulle was added to each tube. Tubes were closed and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1 and 2 were collected for characterization as described in the section E4.a. Parasitic Hymenoptera Bioassay Analytical Phase. In addition, a portion each of Treatment 1 and 2 was aliquoted for use in diets for the sensitive insect bioassay as described in section E4.b. Parasitic Hymenoptera Sensitive Insect Bioassay, and Table 97 and Table 98.

The positive control diet (Treatment 3) was not characterized.

Parasitic Hymenoptera Bioassay

Parasitic Hymenoptera pupae were incubated until adult emergence. Adults were used in the bioassay within 48 hours of emergence.

On Day 0, diet aliquots were removed from frozen storage, uncapped, and distributed into individual plastic cups. One Parasitic Hymenoptera adult was placed in each cup and the cup was sealed with a lid. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for 14

days. Daily, trays were removed from the environmental chamber and missing or dead organisms were recorded. Diet was replaced with new aliquots as described for Day 0 and the trays were returned to the environmental chamber. After 14 days, the bioassay was complete and mortality was assessed. Only cups that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a cup were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4. Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of Parasitic Hymenoptera fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Demonstration of DvSSJ1_210 dsRNA Activity in the *Parasitic Hymenoptera* Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in a sensitive insect bioassay using WCR larvae to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2 of the Parasitic Hymenoptera bioassay. The sensitive insect bioassay was run twice; details regarding both runs of the sensitive insect bioassay are provided in section E4.b. Parasitic Hymenoptera Sensitive Insect Bioassay.

Results and Discussion

The Parasitic Hymenoptera bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 95.

The mortality of Parasitic Hymenoptera fed the test diet containing 1 µg DvSSJ1_210 dsRNA per ml diet (Treatment 2, 6.67%; P-value = 0.9537) was not significantly greater than those fed the bioassay control diet (Treatment 1; 17.2%), as shown in Table 95.

QuantiGene analysis verified the dose and stability under frozen storage conditions of DvSSJ1_210 dsRNA in Treatment 2 and the absence of DvSSJ1_210 dsRNA in Treatment 1. Homogeneity was not analyzed because the diets were solutions.

Observed larval mortality and weight data for the WCR sensitive insect bioassays are summarized in Table 96. The initial WCR bioassay failed to meet the acceptability criterion as the combined dead and missing larval count exceeded 30% in the bioassay control diet (Treatment A) group. A second run of the sensitive insect bioassay also failed to meet the acceptability criterion. However, out-of-study method development records indicated a pattern of high control mortality in WCR fed a diet incorporated with 15% sucrose, the same concentration of sucrose used in diets for the sensitive insect bioassays. In both runs, the biological activity of the DvSSJ1_210 dsRNA in Treatment 2 was verified by increased mortality and decreased weight of WCR exposed to Treatment B compared with Treatment A (Table 96); therefore, the purpose of the sensitive insect bioassay was satisfied and the biological activity of the DvSSJ1_210 dsRNA in Treatment 2 was demonstrated. Details regarding both runs of the sensitive insect bioassay are provided in section E4.b. Parasitic Hymenoptera Sensitive Insect Bioassay.

Conclusion

The results demonstrated that exposure to a concentration of 1 µg DvSSJ1_210 dsRNA per ml diet had no adverse effect on survival of Parasitic Hymenoptera.

Table 95. Summary Analysis of Parasitic Hymenoptera DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (µg DvSSJ1_210 dsRNA/ml)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	29[a]	5	17.2	--
2	Test Diet	1	30	2	6.67	0.9537
3	Positive Control Diet	0[b]	30	30	100	--

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.
[b] Treatment 3 contained a targeted concentration of 8000 µg boric acid per ml.

Table 96. Summary of Parasitic Hymenoptera Sensitive Insect DvSSJ1 dsRNA Bioassay Results

Bioassay	Treatment	Treatment Description	Treatment Dose (ng DvSSJ1_210 dsRNA/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
							Mean ± Standard Deviation	Range
1	A	Bioassay Control Diet	0	29[a]	37.9	18	1.48 ± 0.828	0.2 - 2.7
	B	Test Diet	0.358	25[a]	100	0	NA	NA
2	A	Bioassay Control Diet	0	30	33.3	20	1.75 ± 0.864	0.4 - 3.4
	B	Test Diet	0.358	30	93.3	2	0.500 ± 0.141	0.4 - 0.6

Note: Treatments A and B used in the sensitive insect (WCR) bioassay were prepared from the same stored diet preparations used in Treatments 1 and 2, respectively, of the Parasitic Hymenoptera bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet. Not applicable (NA); there were no surviving WCR in the test diet group.

[a] Organisms counted as missing during the bioassay, or wells containing more than one organism, were not included in the total number of observations for a given treatment.

Table 97. Verification of DvSSJ1_210 dsRNA Dose in Parasitic Hymenoptera Test Diet and Absence in Bioassay Control Diet

Analysis	Treatment	Time Point	% of Expected	Result
Test Diet Dose Verification	2	Day of Preparation	124	Verified[a]
Absence in Bioassay Control Diet	1	Day of Preparation	NA	Verified[b]

Note: Not applicable (NA).

[a] Average of three samples; concentrations within 70-130% of the expected value were considered verified.

[b] Absence of DvSSJ1_210 dsRNA in Treatment 1 was verified by Median Fluorescence Intensity less than the lower limit of quantitation. Three samples were used for absence verification.

Table 98. Verification of Frozen Storage Stability of DvSSJ1_210 dsRNA in Parasitic Hymenoptera Test Diet (Treatment 2)

Time Point	Average % of Day 0 Mean[a]
Day of Diet Preparation[b]	NA
Day 7	106
Day 13	111
Day 21	106
Day 28	108[c]
Day 33	106

Note: Frozen storage stability samples were stored in a -80 °C freezer unit. Not applicable (NA).
[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.
[b] Dose verification samples were used for day of diet preparation assessment of frozen storage stability
[c]Value is the average of two samples. The percentage of Day 0 mean for all other sampling time points is the average of three samples.

E4.a. Parasitic Hymenoptera Bioassay Analytical Phase

QuantiGene analysis was used to verify the dose and the stability under frozen storage conditions of DvSSJ1_210 dsRNA in the test diet (Treatment 2) used in the Parasitic Hymenoptera bioassay and the absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1). The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Carrier and Diets

The 30% sucrose carrier was prepared weight by volume (w/v) with RNase-free water. The test substance was removed from frozen storage and allowed to thaw at room temperature. The test diet (Treatment 2) consisted of carrier spiked with DvSSJ1_210 dsRNA test substance to achieve a concentration of 1 µg DvSSJ1_210 dsRNA per ml diet. The bioassay control diet (Treatment 1) consisted of carrier spiked with the same percent volume RNase-free water as Treatment 2. Both Treatments 1 and 2 were prepared and aliquoted at room temperature.

Characterization of Diets

Sample Collection and Preparation

Immediately following diet preparation, three samples each of Treatment 1 and Treatment 2 were diluted 1:100 in QuantiGene Homogenizing Solution (QHS) and stored frozen (-80 °C freezer unit) until used for DvSSJ1_210 dsRNA absence and dose verification, respectively.

In addition, for assessment of frozen storage stability, aliquots of Treatment 2 were collected during diet aliquoting for the Parasitic Hymenoptera bioassay and stored frozen (-80 °C freezer unit). One aliquot was removed from the freezer approximately weekly for five weeks and diluted 1:100 in QHS (3 replicates per time point). The diluted samples were then stored frozen (-80 °C freezer unit) until analysis, as applicable.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Treatment 1 samples were loaded to the plate at the same dilution as the test diet. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridized to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the Treatment 2 diet was interpolated using the standard curve.

Dose Verification of DvSSJ1_210 dsRNA in Treatment 2

The concentration of DvSSJ1_210 dsRNA in test diet was considered verified if the results fell within 70-130% of the expected value.

Verification of Absence of DvSSJ1_210 dsRNA in Treatment 1

Absence of DvSSJ1_210 dsRNA in Treatment 1 samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean MFI of lowest Standard Curve Point – 10%)

Verification of DvSSJ1_210 dsRNA Stability under Frozen Storage Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean (pg/well) by dividing the individual result for each sample by the mean Day 0 result, multiplied by 100. The percent of means was averaged by time point (Days 7, 13, 21, 28, and 33). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean. For the Day 28 timepoint, two of three samples were used for stability calculation. The remaining sample was excluded because the result was 172% different relative to the average of the other two samples

E4.b. Parasitic Hymenoptera Sensitive Insect Bioassay

The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 used in the Parasitic Hymenoptera bioassay was evaluated by conducting two 14-day bioassays using WCR, a species sensitive to DvSSJ1_210 dsRNA. The initial WCR bioassay was initiated following completion of the Parasitic Hymenoptera bioassay; a second bioassay was conducted when the bioassay control in the first bioassay failed to meet the acceptability criterion.

For each bioassay, WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassays consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc.

WCR larvae in each bioassay were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a diluted portion of Treatment 1)
- Treatment B: Test Diet (containing a diluted portion of Treatment 2 and targeting 0.358 ng DvSSJ1_210 dsRNA per mg *WCR* diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was provided to a target of 30 WCR individuals for each bioassay.

Diet Preparation

Stored aliquots collected from Treatments 1 and 2 during diet preparation for the Parasitic Hymenoptera bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B for each day of diet preparation for each WCR bioassay as follows:

- Treatments 1 and 2 were diluted with RNase-free water to result in dosing solutions containing 15% sucrose.
- Diluted Treatment 1 dosing solution was mixed with carrier in a 2.51:1 ratio (2.51 ml liquid:1 gram of dry diet) to prepare Treatment A.
- Diluted Treatment 2 dosing solution was mixed with carrier in a 2.51:1 ratio to prepare Treatment B.

WCR Bioassays

For each bioassay, WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in a bioassay within 24 hours of hatching. On Day 0 of each bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film and two small holes were poked over each well to allow for ventilation. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassays were complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from statistical analysis.

The bioassay acceptability criterion indicated a bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for each sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 96. Weight data were summarized as means, standard deviations, and ranges for each bioassay and are also presented in Table 96.

E5. Evaluation of the Survival and Development of Green Lacewing Fed an Artificial Diet Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of a meat-based diet prepared by Pioneer Hi-Bred International, Inc.

Positive Control

The positive control consisted of cryolite (AlF_6Na_3).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

The test dosing solution used to prepare Treatment 2 consisted of the test substance diluted in RNase-free water to achieve the concentration in the test diet.

Test System

The test system was green lacewing (Green Lacewing; Neuroptera: Chrysopidae). Green Lacewing was selected as a representative non-target organism to characterize DvSSJ1_210 dsRNA. Green Lacewing eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

Green Lacewing larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet wet weight)
- Treatment 3: Positive Control Diet (targeting 25,000 ng cryolite per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 4 blocks. Each block consisted of a tray containing 1-oz plastic cups and contained 10 replicates from each treatment. Each treatment was fed to a target of 40 Green Lacewing individuals. The

bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed daily and assessed for pupation. On Day 18, all organisms had either pupated or died and the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity and frozen storage stability of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the DvSSJ1_210 dsRNA in Treatment 2.

Bias in the Green Lacewing bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1 and 2 were prepared as described in the Analytical Phase section below. Each dosing solution was mixed with meat-based carrier in a 9:1 ratio (*i.e.*, 9 g carrier to 1 ml dosing solution) to generate Treatments 1 and 2. Treatment 3 was prepared by mixing cryolite with carrier and then combining with RNAse-free water in a 9:1 ratio to a nominal concentration of 25,000 ng cryolite per mg diet wet weight. After mixing, individual diet packets were prepared for each treatment using an encapsulation device and stored frozen (-20 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1 and 2 were collected for characterization of diets as described in Appendix A. In addition, a portion each of Treatment 1 and Treatment 2 was collected for use in diets (Treatments A and B, respectively) for the

sensitive insect bioassay and stored frozen (-20 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in the Sensitive Insect Bioassay section below.

The positive control diet (Treatment 3) was not characterized.

Green Lacewing Bioassay

Green Lacewing eggs were incubated in an environmental chamber until the eggs hatched. Green Lacewing neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet for each treatment was removed from frozen storage. A moisture source (a tube filled with 0.5% agar) was added to each plastic cup utilized in the bioassay. A small hole was poked into each encapsulated diet packet and one thawed diet packet was placed in each plastic cup. One Green Lacewing neonate was placed in each cup containing diet, and then the cup was secured with a lid. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for 18 days. Trays were removed from the environmental chamber and pupated, missing, or dead organisms were recorded daily. Diet packets were replaced daily with new aliquots as described for Day 0 and the trays were returned to the environmental chamber. After 18 days, the bioassay was complete and final mortality was assessed. Only cups that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a cup were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

Green Lacewing fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) (Treatment 2) was greater than the mortality rate of those fed the bioassay control diet (m_C) (Treatment 1). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Rate of Pupation

Rate of pupation was calculated as percentage of pupated insects out of survived insects. Statistical comparison was not conducted due to 100% pupation in both Treatment 1 and Treatment 2.

Demonstration of DvSSJ1_210 dsRNA Activity in the *Green Lacewing* Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay using *WCR* larvae to demonstrate the biological activity of the DvSSJ1_210 dsRNA used in Treatment 2 in the Green Lacewing bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section below.

Results and Discussion

The Green Lacewing bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown inTable 99.

The mortality of Green Lacewing fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 2.50%; P-value = 0.7595) was not significantly greater than those fed the bioassay control diet (Treatment 1; 2.56%), as shown in Table 99. Pupation was 100% in both treatments.

QuantiGene analysis verified the concentration of DvSSJ1_210 dsRNA in the Treatment 2 test dosing solution and the homogeneity and frozen storage stability of DvSSJ1_210 dsRNA in Treatment 2 (Table 102 and Table 103). The absence of DvSSJ1_210 dsRNA in Treatment 1 was assessed in five collected samples. The absence of DvSSJ1_210 dsRNA was verified in two samples but a trace amount was found in the other three samples (Table 102). Treatment 1 samples were analyzed undiluted while Treatment 2 samples were diluted 1:16,000 for analysis. If diluted to the same level as Treatment 2 samples, the DvSSJ1_210 dsRNA signal in the affected Treatment 1 samples would have been approximately 2500 times less than in Treatment 2 and below the assay lower limit of quantitation (LLOQ). Although there were trace amounts of DvSSJ1_210 dsRNA detected in samples of the bioassay control diet, results for Green Lacewing mortality and pupation in this study compared to historical data indicate no effect on the bioassay control diet group response and no impact on this study.

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 101. The WCR bioassay met the acceptability criterion (E5.b. Green Lacewing DvSSJ1 dsRNA Sensitive Insect Bioassay). The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 of the Green Lacewing bioassay was demonstrated by increased

mortality and decreased weight of WCR fed the test diet when compared to those fed the bioassay control diet (Treatment A).

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on mortality or pupation of Green Lacewing.

Table 99. Summary Analysis of Green Lacewing DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	39[a]	1	2.56	--
2	Test Diet	1	40	1	2.50	0.7595
3	Positive Control Diet	0[b]	40	40	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.
[b] Treatment 3 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight.

Table 100. Summary Analysis of Green Lacewing DvSSJ1 dsRNA Bioassay Pupation Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Alive Larvae	Number of Pupated Larvae	Pupation (%)
1	Bioassay Control Diet	0	38	38	100
2	Test Diet	1	39	39	100
3	Positive Control Diet	0[a]	0	-	-

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet wet weight.
[a]Treatment 3 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight.

Table 101. Summary of Green Lacewing Sensitive Insect DvSSJ1 dsRNA Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	30	23.3	23	1.15 ± 0.651	0.2 - 2.6
B	Test Diet	0.2	30	83.3	5	0.380 ± 0.217	0.1 – 0.6

Note: Treatments A and B used in the sensitive insect (WCR) bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 2, respectively, of the Green Lacewing bioassay. The concentration of DvSSJ1_210 dsRNA in Treatment B was based on the wet weight of the artificial diet.

Table 102. Assessment of DvSSJ1_210 dsRNA Concentration in Test Dosing Solution, Homogeneity in Test Diet, and Absence in Bioassay Control Diet

Analysis	Diet Sample	Result
Test Dosing Solution Concentration Verification	NA	Verified[a]
Homogeneity in Test Diet (Treatment 2)	Beginning	Verified[b]
	Middle	
	End	
Absence in Bioassay Control Diet (Treatment 1)	First	Verified[c]
	Second	
	Third	Trace[d]
	Fourth	
	Fifth	

Note: Not applicable (NA). Beginning, middle, and end refer to the stages in the diet aliquoting process at which homogeneity samples were collected.

[a] The test dosing solution concentration was 126% of the expected DvSSJ1_210 dsRNA concentration. Concentrations within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified if the value of each sample was within 70-130% of the mean value of all samples analyzed.

[c] Absence of DvSSJ1_210 dsRNA in Treatment 1 was verified by Median Fluorescence Intensity less than the lower limit of quantitation.

[d] Trace amounts of DvSSJ1_210 dsRNA was detected in three out of five bioassay control samples. The quantity detected is estimated to be approximately 2500-fold less concentrated than in the test diet.

Table 103. Verification of Frozen Storage Stability of DvSSJ1_210 dsRNA in Test Diet

Treatment 2	
Bioassay Stability Day	**Average % of Day 0 Mean[a]**
Day 0	NA
Day 7	99
Day 18	98
Day 26	97
Day 32	94
Day 39	107

Note: Frozen storage stability samples were stored in a -20 °C freezer unit. NA (not applicable).
[a] Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

E5.a. Green Lacewing DvSSJ1 dsRNA Bioassay Analytical Phase

The following dosing solutions were prepared for the Green Lacewing bioassay:

- Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water
- Test dosing solution used to prepare Treatment 2 consisting of test substance diluted in RNase-free water to achieve the concentration in the test diet

QuantiGene analysis was used to verify the concentration of DvSSJ1_210 dsRNA in the test dosing solution and the homogeneity and frozen storage stability of DvSSJ1_210 dsRNA in the test diet (Treatment 2). The absence of DvSSJ1_210 dsRNA in the bioassay control diet (Treatment 1) was also assessed. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation for the Green Lacewing bioassay. To generate the test dosing solution for Treatment 2, the test substance was removed from frozen storage, allowed to thaw at ambient temperature, and then diluted in RNase-free water to the appropriate DvSSJ1_210 dsRNA concentration (10 ng/µl). The bioassay control dosing solution consisted of RNase-free water. Dosing solutions were prepared at room temperature.

Sample Collection and Characterization of Dosing Solutions and Diets

Sample Collection

A sample of the test dosing solution was collected and diluted in QuantiGene Homogenizing Solution (QHS) at a 1:99 dilution ratio (10 µl test dosing solution to 990 µl QHS) on the day of preparation and stored frozen (-80 °C freezer unit). Samples (~50 mg each) of Treatments 1 and 2 were collected into pre-weighed tubes or encapsulated in diet packets during the process of diet aliquoting for the Green Lacewing bioassay, as described in Table 104. The tubes containing diet were weighed again in order to obtain the exact sample weight. Diet samples were stored frozen if applicable (-20 °C freezer unit) until extraction.

Table 104. Green Lacewing Bioassay DvSSJ1 dsRNA Diet Samples

Treatment	Number of Samples	Analysis
1	5	Absence of DvSSJ1_210 dsRNA
2	5 Beginning	Homogeneity of DvSSJ1_210 dsRNA
	5 Middle	
	5 End	
	30[a]	Frozen storage stability

Note: Beginning, middle, and end refer to the stage of the diet aliquoting process at which samples were collected.
[a] Thirty sub-samples were collected for frozen storage stability assessment. Samples for Days 7, 18, 26, 32, and 39 were placed in the freezer (-20 °C freezer unit); samples for Day 0 assessment were not frozen. For each time point, three samples were extracted and two were analyzed.

Preparation of Sample Homogenates

Diet samples were removed from the freezer (if applicable) in preparation for extraction and analysis (three each from the beginning, middle, and end of diet aliquotting for homogeneity, five for Treatment 1, and three for each time point for frozen storage stability).

A volume of QHS (µl) equal to 10x sample weight (mg) was added to each tube containing diet and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were centrifuged again and the final supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis.

QuantiGene Analysis

The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Treatment 1 samples were loaded to the plate undiluted. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the

CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the Treatment 2 dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity and stability under frozen storage conditions), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Treatment 2 Test Dosing Solutio

The concentration of DvSSJ1_210 dsRNA in test dosing solution was considered verified if the result fell within 70-130% of the expected value.

Verification of DvSSJ1_210 dsRNA Homogeneity in Treatment 2

The mean concentration $\overline{x}$ of test substance, expressed in MFI, was determined across all Treatment 2 samples analyzed.

The acceptable range was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in the verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Assessment of Absence of DvSSJ1_210 dsRNA in Treatment 1

Absence of DvSSJ1_210 dsRNA in Treatment 1 samples was verified by MFI results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean MFI of lowest Standard Curve Point – 10%)

A trace amount of DvSSJ1_210 dsRNA was found in one of the 3 samples initially analyzed; therefore, that sample was re-analyzed along with the remaining two Treatment 1 samples collected during diet aliquoting as described in sections B.1., B.2., and B.3. The finding was confirmed for the original sample and a trace amount of DvSSJ1_210 dsRNA was also detected in the additional samples.

Verification of DvSSJ1_210 dsRNA Stability under Frozen Storage Conditions in Treatment 2

Stability was calculated for each time point as a percentage of Day 0 mean MFI by dividing the individual result for each sample by the mean Day 0 MFI result, multiplied by 100. The percent of means was averaged by time point (Days 7, 18, 26, 32, and 39). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

E5.b. Green Lacewing DvSSJ1 dsRNA Sensitive Insect Bioassay

The biological activity of the DvSSJ1_210 dsRNA in Treatment 2 used in the Green Lacewing bioassay was evaluated by conducting a 14-day bioassay using WCR, a species sensitive to DvSSJ1_210 dsRNA. The WCR bioassay was initiated after completion of the Green Lacewing bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)
- Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 0.2 ng DvSSJ1_210 dsRNA per mg *WCR* diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 2 during diet preparation for the Green Lacewing bioassay were removed from the freezer (-20 °C freezer unit) and used to prepare Treatments A and B for each day of diet preparation for the WCR bioassay as follows:

For each respective treatment, RNase-free water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the Green Lacewing bioassay, resulting in a 20% incorporation of the Green Lacewing diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 2 from the Green Lacewing bioassay, resulting in a 20% incorporation of the Green Lacewing diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100 and are presented in Table 101. Weight data were summarized as means, standard deviations, and ranges in Table 101.

E6. Evaluation of the Survival, Weight, and Development of Convergent Lady Beetle Fed an Artificial Diet Containing DvSSJ1 210bp dsRNA

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of *Ephestia* (*Ephestia kuehniella* sp.) eggs.

Test System

The test system was CNV (convergent lady beetle; Coleoptera: Coccinellidae). CNV was selected as a representative coleopteran to characterize the spectrum of activity of DvSSJ1_210 dsRNA. CNV eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

CNV larvae were exposed via oral ingestion to one of the following three treatments:

- Treatment 1: Bioassay Control Diet (prepared with RNase-free water)
- Treatment 2: Test Diet (targeting 1 ng DvSSJ1_210 dsRNA per mg diet dry weight)
- Treatment 3: Positive Control Diet (targeting 15,000 ng boric acid per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a stack of six Petri dishes secured together and contained two replicates from each treatment. Each treatment was fed to a target of 30 CNV individuals. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 17, all organisms had either died or emerged and the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 3) group does not exceed 80%.

Bias was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Diet Preparation and Characterization

The bioassay control diet and test diet (Treatments 1 and 2) were prepared and characterized under a separate study (See Appendix X, section J). A certificate of analysis is included in the study records. The positive control diet (Treatment 3) was also prepared under PHI-2017-127 but was not characterized.

CNV Bioassay

CNV eggs were incubated in an environmental chamber until the eggs hatched. CNV neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet aliquots were removed from frozen storage and approximately 120 mg were aliquoted into individual small caps. A cap containing the appropriate diet treatment and a moisture source (a tube filled with 0.5% agar) were distributed to each Petri dish utilized in the bioassay. One CNV neonate was placed in each dish and the dishes were stacked into blocks and secured together. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Every 3-4 days, bioassay dishes were removed from the environmental chamber, pupation was assessed, and missing or dead organisms were recorded. For each living organism that had not yet pupated or died, old diet was removed from the dish and a new diet-filled cap was prepared and distributed as described for Day 0, prior to returning the dishes to the environmental chamber. Once pupation was observed, organisms were assessed daily for adult emergence. CNV adults were weighed within approximately 24 hours of emergence. On Day 17, all larvae had either died or emerged as adults; therefore, the bioassay was complete and mortality was assessed. Only dishes that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a dish were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparison was made between CNV fed diet containing DvSSJ1_210 dsRNA (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

CNV fed the artificial insect diet containing DvSSJ1_210 dsRNA (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2. Therefore, a two-sample t-test was conducted to test if exposure to DvSSJ1_210 dsRNA caused growth inhibition; i.e., lower weight of CNV fed the artificial insect diet containing DvSSJ1_210 dsRNA (w_T) compared to those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across Treatment 1 and 2 was used. Significance was established if the P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Number of Days to Adult Emergence

The normality assumption necessary for a two-sample *t*-test was not satisfied by data distributions of treatment groups 1 and 2; therefore, a non-parametric two-sample test was conducted to examine if exposure to DvSSJ1_210 dsRNA caused developmental delay.

The corresponding hypothesis test was

$H_0 : F_T(x) = F_c(x)$ *vs.* $H_a : F_T(x) > F_c(x)$

Where $F_T(x)$ is the cumulative distribution of the number of days to adult emergence of CNV fed the artificial insect diet containing DvSSJ1_210 dsRNA, and $F_c(x)$is the cumulative distribution of the number of days to adult emergence of individuals fed the bioassay control diet. Significance would indicate that insects fed the diet containing DvSSJ1_210 dsRNA would have a greater probability to take longer to emerge than those fed the bioassay control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Results and Discussion

The CNV bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 3) group exceeded 80%, as shown in Table 105.

The mortality of CNV fed the test diet containing 1 ng DvSSJ1_210 dsRNA per mg diet (Treatment 2; 7.41%; P-value = 0.6531) was not significantly greater than those fed the bioassay control diet (Treatment 1; 6.67%), as shown in Table 105

The mean weight of CNV fed Treatment 2 (18.0 mg; P-value = 0.1157) was not significantly less than those fed Treatment 1 (19.1 mg), as shown in Table 106.

CNV fed Treatment 2 (median 15 days; Wilcoxon test P-value = 0.3972) and Treatment 1 (median 15 days) did not significantly differ in the probability to take longer to emerge, as shown in Table 3. In addition, the Siegel-Tukey test (P-value = 0.6016) did not show significant evidence that the scales of the two populations differed.

Conclusion

The results demonstrated exposure to a concentration of 1 ng DvSSJ1_210 dsRNA per mg diet had no adverse effect on survival, weight, or adult emergence of CNV.

Table 105. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	2	6.67	--
2	Test Diet	1	27[a]	2	7.41	0.6531
3	Positive Control Diet	0[b]	30	30	100	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight.
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.
[b] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 106. Summary Analysis of CNV DvSSJ1 dsRNA Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Surviving Organisms	Mean Weight (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	28	19.1 (17.8 - 20.3)	14.3 - 26.2	--
2	Test Diet	1	25	18.0 (16.7 - 19.3)	12.8 - 24.5	0.1157
3	Positive Control Diet	0[a]	0	NA	NA	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving *CNV* in Treatment 3.
[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 3. Summary Analysis of CNV Results for Days to Adult Emergence

Treatment	Treatment Description	Treatment Dose (ng DvSSJ1/mg)	Number of Data Points	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel Tukey Test P-Value
1	Bioassay Control Diet	0	28	15.1 ± 0.786	15	13 - 17	--	--
2	Test Diet	1	25	15.2 ± 0.879	15	14 - 17	0.3972	0.6016
3	Positive Control Diet	0[a]	0	NA	NA	NA	--	--

Note: The targeted DvSSJ1_210 dsRNA concentration in Treatment 2 was based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 3.

[a] Treatment 3 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

E7. Northern Bobwhite (*Colinus virginianus*) Acute Oral Toxicity Limit Test with RNA oligonucleotide DvSSJ1_210 dsRNA

MATERIALS AND METHODS

Study Protocol

The methods described in this protocol meet the testing requirements of the OCSPP Guideline 850.2100 (US-EPA, 2012).

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under the stated storage condition. The test substance was received on 16 January 2019 from Pioneer Hi-Bred International, Inc., Johnston, Iowa (Pioneer).

Upon receipt at the contract research organization (CRO), the test solution (No. 20-01) was stored in a freezer (-80 °C) in the original containers.

Determination of stability and characterization, verification of the solution identity, maintenance of records on the solution, and archival of a sample of the solution are the responsibility of Pioneer.

Control Substance

The control substance, nuclease free Invitrogen DEPC-treated water (Control) was received on 20 December 2018 from Life Technologies Corporation, Carlsbad, California.

Upon receipt at The CRO, the control substance (No. 19-36) was stored at room temperature in a dark, ventilated cabinet in the original container.

Test Organism

The northern bobwhite (*Colinus virginianus*) was selected as the test organism since it is recommended by the U.S. EPA and is commonly used as the model of upland gamebirds in toxicity tests of pesticides. A substantial database exists on the response of this species to pesticides. Oral exposure to pesticides is considered appropriate because free-ranging birds may ingest pesticides along with their food items. The northern bobwhite used during this study were obtained from Dewitt's Game Farm, Ellerbe, North Carolina on 21 December 2018. Birds were phenotypically indistinguishable from wild stock. The birds obtained from the supplier were young adults approaching their first breeding season, and were all from the same hatch. Twenty individuals (10 males and 10 females) were randomly selected from the test facility stock colony (No. 18-A-24) for use in the study.

Upon arrival, the test animals were housed in Building 3 House 1, separated by sex, and placed into quarantine. The test animals were acclimated to individual test cages and study room conditions for two weeks prior to testing. The northern bobwhite were 24 weeks and 2 days of age and weighed between 177.2 and 224.8 g at experimental start. There was no mortality observed in the colony during acclimation. The test animals demonstrated no signs of illness or disease before the initiation of the test.

Test Conditions

Housing

Test cage dimensions were 53 × 25.2 × 20.5 to 25 cm. The cages were made of epoxy-coated wire mesh bottoms, fronts, backs, and tops and solid galvanized metal partitions. A solid catch pan was placed beneath each cage and was lined with absorbent paper. Cages were made of non-absorbent materials and were elevated to allow feces to pass through the cage floor, thereby minimizing contact with excreta to minimize disease potential. Feed was provided by aluminum pans. Water was provided by automatic drip waterers with cups. The system was plumbed into the facility's water supply.

Environmental Conditions

Room conditions were maintained according to CRC SOP 11.001. Ventilation within the study room was provided at 15 room exchanges per hour. Daily minimum and maximum temperatures and relative humidity ranges within the test room were monitored daily. During acclimation, study room conditions were maintained at 20 to 28 ℃ and 24 to 54% relative humidity. During the definitive test, study room conditions were maintained at 22 to 26 ℃ and 20 to 56% relative humidity. Temperature was monitored using a digital minimum/maximum thermometer with memory and humidity was monitored using a digital hygrometer.

Lighting within the test room was provided by fluorescent bulbs. The light schedule was set at 10 hours light:14 hours dark, with two 15-minute transition periods, one in the morning and one in the evening. Light intensity averaged 16.5 foot-candles. Light intensity was measured with a digital light meter.

Feed and Water

Purina Gamebird Flight Conditioner was provided during the acclimation and experimental phases of the study. A representative sample from the lot of feed used during this study was analyzed for the presence of pesticides, PCBs, toxic metals, and mycotoxins by Columbia Food Laboratories (Japanese Ministry of Health, Labour and Welfare Registered Laboratory # US20007) and Romer Labs Inc., Union, Missouri with adherence to ISO standards (ISO/IEC

17025: 2005). None of the analytes were detected at concentrations that are considered toxic to the test species. Therefore, the food source was considered to be of acceptable quality.

Water was supplied from a well located at the test facility. Representative samples of the water are analyzed periodically by Columbia Food Labs (Japanese Ministry of Health, Labour and Welfare Registered Laboratory # US20007) with adherence to ISO standards (ISO/IEC 17025: 2005), and Pixis Labs, Portland, Oregon, with adherence to TNI standards, for the presence of pesticides, PCBs, and toxic metals. None of the analytes were detected at concentrations that are considered toxic to the test species. Feed and water were provided to the test organisms *ad libitum*.

Randomization and Control of Bias

Test cages were numbered and randomized using the random number generator in Microsoft Excel. During cage assignment, individual birds were indiscriminately selected from the colony, assigned a unique identification number using Monel #1005-3 aluminum wing tags, and placed individually in test cages following the randomized list until 10 males and 10 females were assigned to test cages. Prior to test initiation, suitable birds were selected from those acclimated, then randomly assigned to test group according to cage number such that five females and five males were designated for each group. A total of 20 birds were used in the study. Cages were labeled with SMV study number, cage number, and group color code.

Dose Preparation and Administration

Dose Levels

Test dose levels were 0 and 105 mg a.i./kg bw.

Dose Preparation

Test substance solution (13.2 mg a.i./mL water) was provided by Pioneer. Control birds were dosed with nuclease-free Invitrogen DEPC-treated water. The test solution was removed from storage the night prior to dosing and thawed in a refrigerator for approximately 15 hours. After the test solution thawed, the test solution was mixed by inverting the vial. The test solution was maintained on wet ice during the dosing procedure.

Dose solution calculations were designed to ensure the appropriate dose was delivered to each bird, ensuring that the total volume of solution delivered to each bird did not exceed 8 mL/kg body weight, and to ensure consistent dosage volumes were delivered to all birds based on body weight. The actual doses delivered to birds are presented in Table 107.

Table 107. DvSSJ1 dsRNA Doses Delivered to Bobwhite Quail

Group[a]		Cage	Sex	ID	Bird Weight (kg)	Target Dose (mg a.i./kg bw)[b]	mg Test Substance/Bird	mg Test Substance/mL Solution (mg/mL)	mL Solution/Bird	mL Solution/kg bw
Control		3	Male	5722	0.1772	0	0	0	1.41	7.95
Control		5	Female	5703	0.1847	0	0	0	1.47	7.95
Control		14	Male	5716	0.2034	0	0	0	1.62	7.95
Control		17	Female	5717	0.1974	0	0	0	1.57	7.95
Control		18	Female	5705	0.1970	0	0	0	1.57	7.95
Control		19	Female	5713	0.2033	0	0	0	1.62	7.95
Control		21	Female	5712	0.2008	0	0	0	1.60	7.95
Control		22	Male	5723	0.2080	0	0	0	1.65	7.95
Control		24	Male	5727	0.2092	0	0	0	1.66	7.95
Control		25	Male	5708	0.1877	0	0	0	1.49	7.95
Test		1	Female	5707	0.2245	105	23.57	13.2	1.79	7.95
Test		4	Female	5719	0.2248	105	23.60	13.2	1.79	7.95
Test		6	Male	5720	0.1995	105	20.95	13.2	1.59	7.95
Test		8	Male	5718	0.2109	105	22.14	13.2	1.68	7.95
Test		9	Male	5710	0.1936	105	20.33	13.2	1.54	7.95
Test		10	Male	5715	0.1818	105	19.09	13.2	1.45	7.95
Test		11	Female	5709	0.2185	105	22.94	13.2	1.74	7.95
Test		12	Female	5724	0.2097	105	22.02	13.2	1.67	7.95
Test		15	Female	5702	0.2111	105	22.17	13.2	1.68	7.95
Test		26	Male	5701	0.1820	105	19.11	13.2	1.45	7.95

[a] Control birds received nuclease free, DEPC-treated water at an equivalent dose volume to the birds receiving the test substance.
[b] Target dose, mg a.i./kg bw = (mg Test Substance/mL Solution × mL Solution/Bird) / Bird Weight (kg)

Dose Administration

On the night prior to Day -1 (day prior to dosing), all birds were fasted during the overnight dark hours. After light hours resumed the following morning (Day -1) acclimated birds were weighed and the weights recorded. Feed was returned to the birds as soon as all weights were recorded. The fasted weights were used to select birds such that the range of body weights

was within ± 10% of the mean gender test population body weight, and that fit a normal distribution with homogeneous variance among groups for use in the study and to calculate individual doses. The test substance solution concentration used to calculate the individual doses was corrected for the purity of the test substance by Pioneer. Cages were randomized to groups and statistical analyses were conducted to confirm that the group assignment did not present significant differences in mean body weights among the groups as assigned.

The birds were again fasted during overnight dark hours the night prior to dosing. The total fasting time of the birds prior to dosing was 15 hours and 40 minutes. All birds were dosed with the control or test substance based on the individual fasted weights taken within 24 hours prior to dosing. The liquid test substance solution was delivered neat, using two 1-mL syringes with 0.01-mL graduations and two 16-guage 3" stainless steel, ball-tipped animal feeding needles to draw and deliver the appropriate volume of test material or water to each bird. Each bird was removed from its respective cage by the assigned bird handler and presented, in the appropriate position, to the individual performing the dosing.

The first of two syringes was inserted into each bird's crop. The syringe plunger was then pressed into the dosing tube, ejecting the syringe contents into the crop of the bird, and the process was immediately repeated with the second syringe. The control-group dosing process was conducted first, followed by the test substance-group dosing process. No regurgitation was observed. The control birds were dosed with nuclease-free DEPC-treated water only. Feed was returned to the test system immediately after the dosing procedures were complete. **Table 108**provides the amount of dose solution, and calculated quantity of test substance, administered to each bird.

Test Monitoring

The birds were dosed at "time 0" (experimental start). Total test duration was 14 days, measured in fourteen 24-hour intervals.

Behavioral Observations

Regurgitation, mortality, general condition, overt signs of toxicity, and abnormal behavior were monitored within the test system. Each animal was carefully observed for 120 consecutive minutes post-dosing, then at three additional time points, which occurred approximately 2, 3.5, and 5 hours post-dosing during the remaining photoperiod on Day 0. Observations were conducted once daily on Days 1, 3, 4, 10, 11, and 14 and twice daily on Days 2, 5 through 9, 12, and 13.

Post-Mortem Examinations

There were no early deaths. All ten surviving birds (five male, five female) from both the control and test substance groups were euthanized by CO_2 asphyxiation and received post-mortem examinations at test termination on Day 14. Post-mortem examinations of gross pathology included evaluation of general physical condition, digestive tract, liver, kidneys, lungs, gall bladder, breast muscles, heart, spleen, and urogenital system.

Body Weight Determination

Body weights were first measured on Day -7. Fasted body weights were measured within 24 hours prior to dosing. Body weights were again measured for each test animal at 7 and 14 days (study termination) post-dosing. Measured body weights were used to calculate change in body weight from Day -1 to Day 7, Day 7 to Day 14, and Day -1 to Day 14 for each animal. Body weight change was calculated as follows, using the Day -1 to Day 7 interval as an example:

(measured body weight Day 7) – (measured body weight Day -1)

Feed Consumption

Feed consumption was measured during the experimental phase for each cage during the following intervals: Day 1 through Day 7 (Week 1) and Day 8 through Day 14 (Week 2). Spillage was primarily prevented by the design of the feed cups used. The total feed consumption for each period and each cage was calculated and divided by the number of days the bird was alive to calculate a mean daily feed consumption per bird. Food consumption per bird per day was calculated as follows, using the Day 1 to Day 7 interval as an example:

$$\frac{\text{(grams food provided Day 1 + added feed) – (grams food remaining Day 7)}}{\text{\# days bird was alive Day 1 to Day 7}}$$

Data Analysis

Pre-treatment body weight data was first tested for normality using Shapiro-Wilk's Test ($\alpha = 0.01$), and for homogeneity of variance among sexes using an F-test ($\alpha = 0.05$; Weber *et al.*, 1989). A t-test ($\alpha = 0.05$; Sokal and Rohlf, 1981; Zar, 1984) was used to test for mean differences among sexes. Pre-treatment body weight data was then tested for homogeneity of variance among groups using an F-test. A t-test was then used to test for mean differences among groups. Mean body weights, calculated body weight change, and weekly feed consumption per bird per day were similarly analyzed at the end of the study. Nonparametric

data was analyzed using the Wilcoxon's Rank Sum (α = 0.05; Mann and Whitney, 1947; Wilcoxon, 1945). Data were statistically analyzed using SAS v9.4 (SAS Institute Inc., 2012).

The nominal oral dose levels tested and the corresponding mortality data derived from the toxicity test were used to assess whether the median lethal dose (LD_{50}) was greater than 105 mg a.i./kg bw, the highest nominal concentration tested.

RESULTS

Behavioral Observations and Mortality

At test termination, 100% survival was recorded for the control group and the test substance group. The LD_{50} was empirically estimated to be >105 mg a.i./kg bw, the highest nominal concentration tested. All birds in the control group and the test substance group were observed to be exhibiting normal behavior throughout the study. The mortality data for this study are presented in Table 109.

Post-Mortem Examinations

No unusual findings were noted among the surviving birds examined at study termination.

Body Weight

Body weights were normally distributed and the data set showed homogenous variance with no statistically significant differences among groups at treatment initiation (p = 0.1627). There were no statistically significant differences among groups in mean body weight on Day 7 (p = 0.1981) or Day 14 (p = 0.2510). There were also no statistically significant differences among groups in mean body weight change from Day -1 to Day 7 (Week 1: p = 0.7452), Day 7 to Day 14 (Week 2: p = 0.4248), or Day -1 to Day 14 (Total: p = 0.4067). Body weight data collected during this study are summarized in Table 110.

Feed Consumption

Average feed consumption per bird per day was significantly higher in birds in the test substance group compared to the control group during Week 1 of the study (p = 0.0229); however, no statistically significant differences were observed in average feed consumption per bird per day during Week 2 of the study ($p = 0.0540$). Feed consumption data collected during this study are summarized in Table 111

ACCEPTABILITY CRITERIA

The following acceptance criteria were required:

Acceptability Criteria	Study Results	Criterion Met
Birds must be randomly assigned to test and control pens.	Birds were randomly assigned to test and control pens.	Yes
During the test, ≤10% of birds in the control group died.	0% of control birds died during the test.	Yes
A minimum of ten birds must be used for the dose level of the test substance and control.	Ten birds were used for the dose level of the test substance and the control.	Yes
Birds must be orally administered the test substance, via either capsule or gavage.	Birds were orally administered the test substance by gavage.	Yes

CONCLUSION

No regurgitation, significant abnormal behaviors, mortalities, or statistically significant differences in mean body weights or mean body weight change between measurement intervals were observed during the study. Average feed consumption per bird per day was significantly higher in birds dosed with 105 mg a.i./kg bw compared to the control during Week 1 of the study (p = 0.0229); however, no statistically significant differences were detected in average feed consumption per bird per day during Week 2 of the study *(p* = 0.0540*).* The LD_{50} was empirically estimated to be >105 mg a.i./kg bw. The No-Observed-Effect Level (NOEL) for mortality was determined to be 105 mg a.i./kg bw. The highest nominal dose level tested, 105 mg a.i./kg bw, was the highest achievable dose level under OCSPP Guideline 850.2100, ensuring that the total volume of solution delivered to each bird did not exceed 8 mL/kg body weight.

Table 108. DvSSJ1_210 dsRNA Northern Bobwhite (*Colinus virginianus*) - Acute Oral Toxicity Test – Doses Administered

Group[a]	Cage	Sex	ID	Bird Weight (kg)	Target Dose (mg a.i./kg bw)[b]	mg Test Substance/ Bird	mg Test Substance/mL Solution (mg/mL)	Actual Dose Volume (mL Solution/Bird)	mL Solution/kg bw
Control	3	Male	5722	0.1772	0	0	0	1.41	7.95
Control	5	Female	5703	0.1847	0	0	0	1.47	7.95
Control	14	Male	5716	0.2034	0	0	0	1.62	7.95
Control	17	Female	5717	0.1974	0	0	0	1.57	7.95
Control	18	Female	5705	0.1970	0	0	0	1.57	7.95
Control	19	Female	5713	0.2033	0	0	0	1.62	7.95
Control	21	Female	5712	0.2008	0	0	0	1.60	7.95
Control	22	Male	5723	0.2080	0	0	0	1.65	7.95
Control	24	Male	5727	0.2092	0	0	0	1.66	7.95
Control	25	Male	5708	0.1877	0	0	0	1.49	7.95
Test	1	Female	5707	0.2245	105	23.57	13.2	1.79	7.95
Test	4	Female	5719	0.2248	105	23.60	13.2	1.79	7.95
Test	6	Male	5720	0.1995	105	20.95	13.2	1.59	7.95
Test	8	Male	5718	0.2109	105	22.14	13.2	1.68	7.95
Test	9	Male	5710	0.1936	105	20.33	13.2	1.54	7.95
Test	10	Male	5715	0.1818	105	19.09	13.2	1.45	7.95
Test	11	Female	5709	0.2185	105	22.94	13.2	1.74	7.95
Test	12	Female	5724	0.2097	105	22.02	13.2	1.67	7.95
Test	15	Female	5702	0.2111	105	22.17	13.2	1.68	7.95
Test	26	Male	5701	0.1820	105	19.11	13.2	1.45	7.95

[a] Control birds received nuclease free, DEPC-treated water at an equivalent dose volume to the birds receiving the test substance.

[b] Target dose, mg a.i./kg bw = (mg Test Substance/mL Solution × mL Solution/Bird) / Bird Weight (kg)

Table 109. DvSSJ1_210 dsRNA Northern Bobwhite (*Colinus virginianus*) - Acute Oral Toxicity Test - Survival

Dose	0 mg a.i./kg bw	105 mg a.i./kg bw
Study Day	**% Surviving**	
0	100	100
1	100	100
2	100	100
3	100	100
4	100	100
5	100	100
6	100	100
7	100	100
8	100	100
9	100	100
10	100	100
11	100	100
12	100	100
13	100	100
14	100	100

Table 110. DvSSJ1_210 dsRNA Northern Bobwhite (*Colinus virginianus*) Acute Oral Toxicity Test – Body Weight

Measurement Interval	Average Body Weight (g) (SD) by Dose	
	0 mg a.i./kg bw	105 mg a.i./kg bw
Day -7	201.5	208.9
	(11.1)	(17.5)
Day -1	196.9	205.6
	(10.5)	(15.9)
Day 7	200.0	208.0
	(11.6)	(14.9)
Change in Weight - Day -1 to Day 7	3.2	2.4
	(4.5)	(6.0)
Day 14	200.2	206.5
	(11.5)	(12.6)
Change in Weight - Day 7 to Day 14	0.1	-1.5
	(0.9)	(4.7)
Change in Weight - Day -1 to Day 14	3.3	0.9
	(5.0)	(7.3)

SD = Standard Deviation

Table 111. DvSSJ1_210 dsRNA Northern Bobwhite (*Colinus virginianus*) Acute Oral Toxicity Test – Feed Consumption

Measurement Interval	Average Feed Consumption (g) (SD) by Dose	
	0 mg a.i./kg bw	**105 mg a.i./kg bw**
Week 1	14.2	16.4[a]
	(1.59)	(2.27)
Week 2	12.6	13.8
	(1.39)	(1.25)

[a] Significantly higher compared to the control, based on a t-test.

SD = Standard Deviation

Appendix F. IPD072Aa Protein Spectrum of Activity Bioassay Materials and Methods

F1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C

Carrier

The carrier consisted primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2-8 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

Test System

The test system was *Diabrotica virgifera virgifera* (western corn rootworm; Coleoptera: Chrysomelidae; WCR). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

Multiple independent dose-response bioassays were conducted exposing WCR larvae via oral ingestion to the following eight treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 2 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 4 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 8 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Test Diet (targeting 16 ng IPD072Aa protein per mg diet wet weight)
- Treatment 6: Test Diet (targeting 32 ng IPD072Aa protein per mg diet wet weight)
- Treatment 7: Test Diet (targeting 64 ng IPD072Aa protein per mg diet wet weight)
- Treatment 8: Test Diet (targeting 128 ng IPD072Aa protein per mg diet wet weight)

Within each bioassay, treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3 replicates from each treatment. Each treatment in each bioassay was fed to a target of 30 WCR individuals. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 3 or Day 4. After each 7-day bioassay, mortality was assessed, and surviving organisms were individually weighed.

For each bioassay, a quantitative enzyme-linked immunosorbent assay (ELISA) method was used to verify the concentration of IPD072Aa protein in the working stock solutions used to prepare the dosing solutions for Treatments 2-8. The homogeneity of IPD072Aa protein in two diets per bioassay and the stability under bioassay conditions of Treatment 2 was also verified.

The bioassay acceptability criterion indicated a bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment 1) group.

Bias in the WCR bioassays was controlled through the randomization of treatments within blocks and the use of control diet. Bias in the characterization portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptability criteria. In addition, the use of working stock solutions and stabilized curve preparation minimized day to day bias.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

On each day of diet preparation for each bioassay, dosing solutions were individually prepared as described in the Analytical Phase Section below. Dosing solutions for Treatments 2-8 were prepared from IPD072Aa protein working stock solutions; Treatment 1 dosing solutions consisted of ultrapure water. Each dosing solution was mixed with carrier in a 2.51:1 ratio (*i.e.*, 2.51 ml of dosing solution to 1 g of carrier), generating Treatments 1-8.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1-8 were collected for characterization as described in the Analytical Phase Section below.

WCR Bioassay

Bioassays were conducted to determine the response of WCR to IPD072Aa protein exposure via oral ingestion. A separate batch of WCR eggs for each bioassay was incubated in an environmental chamber until the eggs hatched. WCR neonates were used in each bioassay within 24 hours of hatching.

On Day 0 of each respective bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One WCR neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for a total of 7 days. On Day 3 or Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from statistical analysis.

Statistical Analysis

Statistical analyses were conducted using SAS software, Version 9.4, to estimate the median lethal concentration (LC_{50}) of IPD072Aa protein in artificial insect diet for WCR.

For each of the four independent bioassays, a log-logistic regression model was utilized to analyze the dose-response curve data. Let y_{ijk} indicate the mortality status of the k^{th} insect exposed to the j^{th} protein concentration x_j in the i^{th} bioassay, where i = 2, 3, 4, 5; j = 1, …, 8; k = 1, …, 30; and y_{ijk} = 1 if the insect was dead at the end of the experiment and y_{ijk} = 0 if the insect was alive. Upon applying Abbott's correction, y_{ijk} was assumed to follow Bernoulli (c_i + (1 - c_i)π_{ij}), where c_i denotes the baseline mortality rate in the i^{th} bioassay and π_{ij} denotes the mortality rate caused by exposure to protein concentration x_j in the i^{th} bioassay. If the observed baseline mortality rate was zero in the i^{th} bioassay, then c_i was fixed at zero. The "logit" function was expressed as

$$\text{logit}(\pi_{ij}) = \ln(\frac{\pi_{ij}}{1-\pi_{ij}}),$$

which transformed the parameter π_{ij} on the probability scale into a parameter on the linear predictor scale. The regression model on the linear predictor scale was

$$\text{logit}(\pi_{ij}) = \beta_i \log_{10}(\frac{x_j}{\theta_i}),$$

where β_i denotes the slope and θ_i corresponds to the LC_{50} in the i^{th} bioassay.

SAS PROC NLMIXED was used to fit the log-logistic regression model for each bioassay. Model parameter estimates and their standard errors (SE) were obtained using the maximum likelihood method.

A random-effects meta-analysis approach (Normand, 1999) was utilized in SAS PROC MIXED to derive the estimate of the overall mean LC_{50}, $\hat{\theta}$, across the independent bioassays:

$$\hat{\theta} = \frac{\sum_{i=1}^{4} W_i \hat{\theta}_i}{W_i} \; with \; W_i = \frac{1}{\hat{S}_i^{\,2} + \hat{\tau}^2}$$

where $\hat{\theta}_i$ represents the estimate of θ_i for the i^{th} bioassay, $\hat{s}_i$ represents the estimate of SE for $\hat{\theta}_i$, and $\hat{\tau}^2$ represents the estimate of the variation of LC_{50} between bioassays. The estimated SE of $\hat{\theta}$ was $(\sum_{i=1}^{4} W_i)^{-\frac{1}{2}}$.

SAS PROC MIXED was utilized to obtain the estimate and SE of the overall mean LC_{50}.

The LC_{50} point estimate, SE, and 95% confidence interval for each individual bioassay as well as the overall means are reported in **Table 112**.

Results and Discussion

The acceptability criterion for the WCR bioassays indicated a bioassay could be terminated and repeated if the combined number of dead and missing organisms exceeded 30% in the bioassay control diet (Treatment 1). Bioassay 1 did not meet the acceptability criterion and was terminated. Bioassays 2, 3, 4, and 5 met the acceptability criterion and those results are reported. Details regarding the terminated bioassay will not be reported but will be maintained in the study records.

The LC_{50} point estimate, SE, and 95% confidence interval for each individual bioassay, as well as the overall means derived by meta-analysis across the four bioassays, are reported in Table 112. The estimated overall mean LC_{50} for WCR was 26 ng IPD072Aa protein per mg diet with a 95% confidence interval of 16-37 ng IPD072Aa protein per mg diet (Table 112).

For each bioassay, an ELISA method verified the concentration of IPD072Aa protein in the working stock solutions used to prepare the dosing solutions for Treatments 2-8 (Table 114). The homogeneity of IPD072Aa protein in two diets per bioassay and the stability under bioassay conditions of Treatment 2 were also verified (Table 115 and Table 116). Homogeneity assessment of two test diets per bioassay on one day of diet preparation each is considered representative of both days of diet preparation for all test diets in each bioassay.

A summary of WCR larval mortality data is provided in Table 113. A summary of WCR larval weight data is provided in Table 118.

Conclusion

The estimated overall mean LC_{50} for WCR was 26 ng IPD072Aa protein per mg diet wet weight with a 95% confidence interval of 16-37 ng IPD072Aa protein per mg diet.

Table 112. Median Lethal Concentration of IPD072Aa Protein for WCR

Bioassay	LC_{50}		
	Point Estimate	Standard Error	95% Confidence Interval
2	12	3.9	4.1 - 19
3	39	4.7	29 - 48
4	30	4.5	21 - 39
5	26	3.9	19 - 34
Overall Mean[a]	26	5.6	16 - 37

Note: Median lethal concentration (LC_{50}) is expressed in ng IPD072Aa protein per mg diet wet weight.
[a] The overall mean values as determined with the statistical analysis described in the Methods section.
Note: Standard error (SE).

Table 113. Summary Analysis of WCR IPD072Aa Protein Bioassay Larval Mortality

Bioassay	Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
2	1	Bioassay Control Diet	27[a]	1	3.70
	2	Test Diet (2 ng IPD072Aa/mg)	29[a]	8	27.6
	3	Test Diet (4 ng IPD072Aa/mg)	30	7	23.3
	4	Test Diet (8 ng IPD072Aa/mg)	28[a]	11	39.3
	5	Test Diet (16 ng IPD072Aa/mg)	27[a]	15	55.6
	6	Test Diet (32 ng IPD072Aa/mg)	28[a]	25	89.3
	7	Test Diet (64 ng IPD072Aa/mg)	28[a]	26	92.9
	8	Test Diet (128 ng IPD072Aa/mg)	27[a]	27	100
3	1	Bioassay Control Diet	27[a]	4	14.8
	2	Test Diet (2 ng IPD072Aa/mg)	30	1	3.33
	3	Test Diet (4 ng IPD072Aa/mg)	26[a]	5	19.2
	4	Test Diet (8 ng IPD072Aa/mg)	30	4	13.3
	5	Test Diet (16 ng IPD072Aa/mg)	27[a]	7	25.9
	6	Test Diet (32 ng IPD072Aa/mg)	26[a]	10	38.5
	7	Test Diet (64 ng IPD072Aa/mg)	29[a]	26	89.7
	8	Test Diet (128 ng IPD072Aa/mg)	28[a]	28	100
4	1	Bioassay Control Diet	29[a]	0	0
	2	Test Diet (2 ng IPD072Aa/mg)	29[a]	2	6.90

Bioassay	Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
	3	Test Diet (4 ng IPD072Aa/mg)	29[a]	2	6.90
	4	Test Diet (8 ng IPD072Aa/mg)	29[a]	5	17.2
	5	Test Diet (16 ng IPD072Aa/mg)	30	8	26.7
	6	Test Diet (32 ng IPD072Aa/mg)	30	17	56.7
	7	Test Diet (64 ng IPD072Aa/mg)	30	20	66.7
	8	Test Diet (128 ng IPD072Aa/mg)	28[a]	25	89.3
5	1	Bioassay Control Diet	29[a]	3	10.3
	2	Test Diet (2 ng IPD072Aa/mg)	29[a]	3	10.3
	3	Test Diet (4 ng IPD072Aa/mg)	28[a]	2	7.14
	4	Test Diet (8 ng IPD072Aa/mg)	29[a]	5	17.2
	5	Test Diet (16 ng IPD072Aa/mg)	29[a]	10	34.5
	6	Test Diet (32 ng IPD072Aa/mg)	30	21	70.0
	7	Test Diet (64 ng IPD072Aa/mg)	30	23	76.7
	8	Test Diet (128 ng IPD072Aa/mg)	30	29	96.7

Note: Targeted IPD072Aa protein concentrations in Treatments 2-8 were based on diet wet weight.

[a] Organisms counted as missing during the bioassay, or wells containing more than one organism, were not included in the total number of observations for a given treatment.

Table 114. Verification of IPD072Aa Protein WCR Bioassay Working Stock Solutions Concentrations

Bioassay	Time Point	Target Concentration (µg/ml)	Mean ELISA Result (µg/ml)	% of Target[a]	Result
2	Day 0	179	170	95.1	Verified[a]
	After final use		174	97.0	
3	Day 0		173	96.4	
	After final use		170	95.0	
4	Day 0		188	105	
	After final use		172	95.8	
5	Day 0		189	106	
	After final use		182	102	

Note: Quantitative enzyme-linked immunosorbent assay (ELISA).

[a] Working stock solutions concentrations within 70-130% of the expected value were considered verified.

Table 115. Verification of IPD072Aa Protein Homogeneity in WCR Bioassay Test Diets

Bioassay	Treatment	Mean ELISA Result (ng/ml)	-30% (ng/ml)	+30% (ng/ml)	Sample Minimum	Sample Maximum	Homogeneity
2	4	432	302	561	390	466	Verified
	6	1968	1378	2559	1851	2122	Verified
3	7	4029	2820	5237	3731	4372	Verified
	8	8052	5636	10467	7779	8484	Verified
4	2	102	71	132	96	107	Verified
	3	210	147	273	193	232	Verified
5	4	476	333	619	437	522	Verified
	5	977	684	1270	895	1082	Verified

Note: Homogeneity was considered verified for a given bioassay and treatment if the value of each sample was within 70-130% of the
mean value of all samples analyzed for that bioassay and treatment.

Table 116. Verification of IPD072Aa Protein Stability under WCR Bioassay Conditions in Treatment 2

Time Point	Adjusted Result ng/ml	CV	Average Adjusted Result ng/ml	% of Day 0	Stability
Day 0	90.26	2.2	91.3	NA	NA
	93.57				
	90.08				
Day 1	84.16	1.7	83.4	91.3	Verified
	84.26				
	81.78				
Day 2	76.14	4.3	80.1	87.7	Verified
	81.42				
	82.59				
Day 3	85.46	6.1	81.1	88.8	Verified
	82.05				
	75.70				
Day 4	76.94	4.6	76.3	83.6	Verified
	79.47				
	72.53				

Note: Coefficient of variation (CV). Not applicable (NA). Diet samples were considered stable if the average percentage for each sampling time point was ≥ 70% of the Day 0 mean.

F1.a. Western Corn Rootworm IPD072Aa Protein Bioassay Analytical Phase

The following dosing solutions were prepared for the WCR bioassays:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure water

Test dosing solutions used to prepare Treatments 2-8 consisting of IPD072Aa test substance diluted in ultrapure water to achieve the concentrations in the test diets.

For each bioassay, a quantitative enzyme-linked immunosorbent assay (ELISA) method was used to verify the concentration of IPD072Aa protein in the working stock solutions used to prepare the dosing solutions for Treatments 2-8. The homogeneity of IPD072Aa protein in two diets per bioassay and the stability under bioassay conditions of Treatment 2 were also verified.

Preparation of Solutions

Aliquots of the test substance were thawed under chilled conditions, pooled together (as applicable), and then diluted in ultrapure water to create IPD072Aa protein working stock solutions (targeted concentration 179 ng/µl) for the bioassays. On each day of diet preparation, working stock solution was diluted in ultrapure water to prepare the dosing solutions for Treatments 2-7 and used directly to prepare the dosing solution for Treatment 8. The IPD072Aa protein concentrations in the test dosing solutions ranged from 2.80-179 ng/µl to

achieve the desired concentrations in Treatments 2-8. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were prepared and maintained chilled.

Characterization of IPD072Aa Protein Working Stock Solutions

The concentration of IPD072Aa protein in each working stock solution was verified for the day of solubilization and following final use using a quantitative ELISA method that had been internally validated to demonstrate method suitability. Samples were interpolated using a standard curve with the IPD072Aa test substance as the analytical standard.

IPD072Aa Protein ELISA Method

Prior to analysis, samples were diluted as applicable in chilled 25% StabilZyme Select in phosphate-buffered saline containing polysorbate 20 (PBST). Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with an IPD072Aa -specific antibody. Following incubation, unbound substances were washed from the plate. A different IPD072Aa-specific antibody, conjugated to the enzyme horseradish peroxidase (HRP), was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound IPD072Aa-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader.

Determination of Protein Concentration

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the OD values obtained for each set of sample wells to a protein concentration value.

A standard curve was included on each ELISA plate. The equation for the standard curve was derived by the software, which used a quadratic fit to relate the OD values obtained for each set of standard wells to the respective standard concentration (ng/ml).

The quadratic regression equation was applied as follows: $y = Cx^2 + Bx + A$

where x = known standard concentration and y = respective absorbance value (OD)

Interpolation of the sample concentration (ng/ml) was performed by solving for x in the above equation using the values for A, B, and C that were determined for the standard curve.

Sample Concentration (ng/ml) = $\frac{-B + \sqrt{B^2 - 4C(A - \text{sample OD})}}{2C}$

For example, given curve parameters of A = 0.0476, B = 0.4556, C= -0.01910, and a sample OD = 1.438

Sample Concentration = $\frac{-0.4556 + \sqrt{0.4556^2 - 4(-0.01910)(0.0476 - 1.438)}}{2(-0.01910)}$ = 3.6 ng/ml

The sample concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the interpolated concentration by N.

Adjusted Concentration = Interpolated Sample Concentration x Dilution Factor

For example, given an interpolated concentration of 3.6 ng/ml and a dilution factor of 1:20

Adjusted Concentration = 3.6 ng/ml x 20 = 72 ng/ml

Characterization of Diets

Sample Collection

During the process of diet distribution for the WCR bioassays, samples were collected as shown in the following table:

Table 117. WCR IPD072Aa Bioassay Diet Collection

Analysis	Bioassay	Treatments	Number of Samples
Homogeneity of IPD072Aa protein	2	4, 6	15 per treatment[a]
	3	7, 8	15 per treatment[a]
	4	2, 3	15 per treatment[a]
	5	4, 5	15 per treatment[a]
Stability under bioassay conditions	2	2	25[b]
Oven dry weight equivalence	2	1	25[b]

Note: Homogeneity assessment of two test diets per bioassay on one day of diet preparation each is considered representative of both days of diet preparation for all test diets in each bioassay.

[a] Five samples were collected at the beginning of the diet distribution process, five in the middle, and five at the end for each homogeneity analysis.

[b] Five samples were collected for Day 0 analysis; the remaining samples were distributed to bioassay plates and placed under bioassay conditions.

In addition, a sample of Treatment 1 was collected from each diet preparation and stored frozen (-80 °C freezer unit. Treatment 1 samples were not analyzed.

Stability Under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

Samples were collected during one day of diet preparation for verification of stability of IPD072Aa protein in Treatment 2 under bioassay conditions. For the Day 0 assessment, five samples each of Treatment 1 and 2 were collected in preparation for ELISA analysis. For Days 1-4 assessment, samples of Treatment 1 and Treatment 2 (five each per day) were randomly distributed to bioassay plates and placed under bioassay conditions using the same methods and conditions used in the *WCR* bioassay, except they were not infested with larvae. For each day of stability analysis (Days 1-4), five samples each of Treatment 1 and 2 were removed from bioassay conditions. Treatment 2 samples were sub-sampled (40 mg) and stored frozen (-80 °C freezer unit) until analysis. Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Preparation of Sample Homogenates

Collected diet samples for homogeneity (nine homogeneity samples per treatment; three each from the beginning, middle, and end of diet preparation for each assessment) and three Treatment 2 Day 0 bioassay stability samples were weighed to approximately 40 mg on wet ice in preparation for extraction and analysis. In addition, weighed Treatment 2 bioassay stability samples for Days 1-4 were removed from the freezer in preparation for extraction and analysis. For assessment of homogeneity and Day 0 stability under bioassay conditions, sub-samples were extracted in 600 µl of 25% StabilZyme Select in chilled PBST. For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 25% StabilZyme Select in chilled PBST sample buffer with volumes normalized depending on the average ODE for each time point. Homogeneity and stability analysis was conducted using the previously described method with the following exceptions for calculations.

Homogeneity Assessment

The mean concentration $\overline{x}$ of test substance, expressed in ng/ml, was determined across all samples analyzed for a given test diet.

The acceptable range was calculated, using the following equation:

Acceptable range = $\overline{x}$ × (1 ± 0.3)

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Stability under Bioassay Conditions Assessment

Stability was calculated for each time point as a percentage of Day 0 mean concentration, expressed in ng/ml, by dividing the individual result for each sample by the mean Day 0 result, multiplied by 100. The percent of means was averaged by sampling day (Days 1-4). Stability was considered verified if the average percentage for a sampling time point was ≥ 70% of the Day 0 mean.

Table 118. Summary of WCR IPD072Aa Bioassay Larval Weight Data

Bioassay	Treatment	Treatment Description	Number of Surviving Organisms	Weight (mg)		
				Mean	Standard Deviation	Range
2	1	Bioassay Control Diet	26	0.442	0.124	0.1 - 0.7
	2	Test Diet (2 ng IPD072Aa/mg)	21	0.286	0.101	0.1 - 0.4
	3	Test Diet (4 ng IPD072Aa mg)	23	0.213	0.0869	0.1 - 0.4
	4	Test Diet (8 ng IPD072Aa/mg)	17	0.135	0.0606	0.1 - 0.3
	5	Test Diet (16 ng IPD072Aa/mg)	12	0.0917	0.0515	0 - 0.2
	6	Test Diet (32 ng IPD072Aa/mg)	3	0.100	0	0.1 - 0.1
	7	Test Diet (64 ng IPD072Aa mg)	2	0.0500	0.0707	0 - 0.1
	8	Test Diet (128 ng IPD072Aa/mg)	0	NA	NA	NA
3	1	Bioassay Control Diet	23	0.426	0.160	0.1 - 0.7
	2	Test Diet (2 ng IPD072Aa/mg)	29	0.366	0.126	0.1 - 0.6
	3	Test Diet (4 ng IPD072Aa/mg)	21	0.233	0.115	0.1 - 0.4
	4	Test Diet (8 ng IPD072Aa/mg)	25	0.136	0.0490	0.1 - 0.2
	5	Test Diet (16 ng IPD072Aa/mg)	20	0.0950	0.0224	0 - 0.1
	6	Test Diet (32 ng IPD072Aa/mg)	16	0.0875	0.0342	0 - 0.1
	7	Test Diet (64 ng IPD072Aa/mg)	3	0.0667	0.0577	0 - 0.1
	8	Test Diet (128 ng IPD072Aa/mg)	0	NA	NA	NA
4	1	Bioassay Control Diet	29	0.472	0.165	0.1 - 0.9
	2	Test Diet (2 ng IPD072Aa/mg)	27	0.356	0.125	0.2 - 0.6
	3	Test Diet (4 ng IPD072Aa/mg)	27	0.274	0.120	0 - 0.5
	4	Test Diet (8 ng IPD072Aa/mg)	23	0.196	0.122	0.1 - 0.5
	5	Test Diet (16 ng IPD072Aa/mg)	22	0.141	0.0590	0.1 - 0.3
	6	Test Diet (32 ng IPD072Aa/mg)	13	0.0923	0.0494	0 - 0.2
	7	Test Diet (64 ng IPD072Aa/mg)	10	0.170	0.106	0 - 0.3
	8	Test Diet (128 ng IPD072Aa/mg)	3	0.0667	0.0577	0 - 0.1
5	1	Bioassay Control Diet	26	0.496	0.140	0.1 - 0.7
	2	Test Diet (2 ng IPD072Aa/mg)	26	0.431	0.132	0.2 - 0.6
	3	Test Diet (4 ng IPD072Aa/mg)	26	0.312	0.103	0.1 - 0.6
	4	Test Diet (8 ng IPD072Aa/mg)	24	0.221	0.118	0.1 - 0.6
	5	Test Diet (16 ng IPD072Aa/mg)	19	0.184	0.0958	0.1 - 0.4
	6	Test Diet (32 ng IPD072Aa/mg)	9	0.122	0.0441	0.1 - 0.2
	7	Test Diet (64 ng IPD072Aa/mg)	7	0.114	0.0378	0.1 - 0.2
	8	Test Diet (128 ng IPD072Aa/mg)	1	0.1	NC[a]	NC

Note: Targeted IPD072Aa protein concentrations in Treatments 2-8 are based on diet wet weight. Not applicable (NA); there were no surviving *Diabrotica virgifera virgifera* in Bioassays 2 and 3, Treatment 8.

[a] The reported mean is the weight value of the one surviving organism and the standard deviation and range are not calculated (NC

F2. Evaluation of the Survival and Weight of Southern Corn Rootworm Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted primarily of Stonefly Heliothis diet.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in Appendix A.

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was SCR (Southern corn rootworm; Coleoptera: Chrysomelidae). SCR was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. SCR eggs were obtained from Crop Characteristics, Inc. (Farmington, MN, USA) and identity was recorded by study personnel.

Experimental Design

SCR larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 10,000 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3 replicates from each treatment. Each treatment was fed to a target of 30 SCR individuals. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 4. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatment 2 and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatment 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4 of the SCR bioassay.

Bias in the SCR bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Bulk dosing solutions for Treatments 1-5 were prepared and maintained as described in The Analytical Phase section below. The boric acid dosing solution for Treatment 6 was prepared on each day of diet preparation by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 10,000 ng/mg diet wet weight and maintained chilled (in a refrigerator set at 4 °C or on wet ice) until use. On each day of diet preparation, each dosing solution was mixed with carrier in a 2.51:1 ratio (i.e., 2.51 ml of dosing solution to 1 g of carrier), generating Treatments 1-6.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1, 2, 4, and 5 were collected for characterization of diets as described in the Analytical Phase section below. In addition, a portion of Treatment 1 and Treatment 4 from each day of diet preparation was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay as described in the Sensitive Insect Bioassay section below.

Treatment 3 and the positive control diet (Treatment 6) were not characterized.

SCR Bioassay

SCR eggs were incubated in an environmental chamber until the eggs hatched. SCR neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One SCR neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for a total of 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living SCR larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. One organism from Treatment 4 was lost during weighing so only mortality was recorded for that individual. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparisons were made between SCR fed diet containing IPD072Aa protein (Treatments 2, 3, 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

SCR fed an artificial diet containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The distributions of weight were evaluated for each treatment. The data were not normally distributed; therefore, non-parametric two-sample tests were conducted separately to examine if exposure to IPD072Aa protein of each concentration caused growth inhibition compared to exposure to the bioassay control diet. The corresponding hypothesis test was

$$H_0 : F_T(x) = F_c(x) \ \ vs. \ H_a : F_T(x) < F_c(x)$$

Where $F_T(x)$ is the cumulative distribution of the weight of *SCR* fed the artificial diet containing IPD072Aa protein, and $F_c(x)$ is the cumulative distribution of the weight of individuals fed the bioassay control diet. Therefore, significance would indicate that insects fed the diet containing IPD072Aa protein would have a greater probability to weigh less than those fed the control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Demonstration of IPD072Aa Protein Activity in a SCR Test Diet

A portion of Treatments 1 and 4 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 4 in the SCR bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section.

Results and Discussion

The SCR bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 30% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in **Table 119**.

The mortality of SCR fed the test diets containing 100 and 500 ng/mg IPD072Aa protein (Treatments 2 and 3; 3.33% and 17.2%; P-values = 0.7542 and 0.0896; respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1; 3.33%), as shown in Table 1. The mortality of SCR fed the test diet containing 1000 ng/mg IPD072Aa protein (Treatment 4; 25.0%; P-value = 0.0202) was significantly greater than those fed Treatment 1, as shown inTable 119.

The SCR fed Treatments 2, 3 and 4 (medians of 0.3 mg, 0.2 mg, and 0.2 mg, respectively; Wilcoxon test P-values < 0.0001) had significantly greater probability of weighing less than those fed Treatment 1 (median 0.8 mg), as shown in Table 2. Since the Siegel-Tukey test (P-value 0.9387, 0.2509, and 0.0546 for Treatments 2, 3, and 4, respectively) did not show significant evidence that the scales of the populations differed, as shown in Table 2, the significant difference identified with the Wilcoxon test can be interpreted as a difference in medians.

Western blot analysis visually confirmed the dose and the homogeneity (Figure 7, Figure 8, and Figure 9) of the IPD072Aa protein in Treatments 2 and 4. Homogeneity assessment of two test diets on one day of diet preparation each was considered representative of both days of diet preparation for all test diets. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed (Figure 9). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 3); however, the band was less intense than that of the test diets (Treatments 2 and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 3). The stability under bioassay conditions of the IPD072Aa protein in Treatment 2 (Figure 10) was also assessed. Some variability was observed within the Day 0 and Day 1 replicate samples, likely due to western blot transfer issues. The overall trend showed similar band intensity throughout the four days tested.

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 3. The WCR bioassay met the acceptability criterion (D.1.a. WCR DvSSJ1 dsRNA Bioassay Analytical Phase). The biological activity of the IPD072Aa protein in Treatment 4 of the SCR bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in Table 121 and section F2.b. Southern Corn Rootworm IPD072Aa Protein Sensitive Insect Bioassay) when compared to the bioassay control diet.

Conclusion

The results demonstrated the mortality for SCR fed the test diets containing 100 ng IPD072Aa protein per mg diet (Treatment 2; 3.33%) and 500 ng IPD072Aa protein per mg diet (Treatment 3; 17.2%) was not significantly greater than the mortality for SCR fed the bioassay control diet (Treatment 1; 3.33%). A statistically significant difference was observed in mortality between SCR fed the test diet containing 1000 ng IPD072Aa protein per mg diet (Treatment 4; 25.0%) and those fed Treatment 1. In addition, statistically significant differences were observed in median weight between SCR fed Treatments 2, 3 and 4 (0.3 mg, 0.2 mg, and 0.2 mg, respectively) and those fed Treatment 1 (0.8 mg).

Table 119. Summary Analysis of SCR IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	30	1	3.33	0.7542
3	Test Diet	500	29[a]	5	17.2	0.0896
4	Test Diet	1000	28[a]	7	25.0	0.0202[b]
5	Heat-treated Control Diet	1000	30	3	10.0	--
6	Positive Control Diet	0[c]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed.

[c] Treatment 6 contained a targeted concentration of 10,000 ng boric acid per mg diet wet weight.

Table 120. Summary Analysis of SCR IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Median (mg)	Mean Weight ± Standard Deviation (mg)	Range (mg)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	29	0.8	0.862 ± 0.235	0.5 - 1.4	--	--
2	Test Diet	100	29	0.3	0.300 ± 0.0926	0.2 - 0.6	<0.0001[a]	0.9387
3	Test Diet	500	24	0.2	0.192 ± 0.0584	0.1 - 0.3	<0.0001[a]	0.2509
4	Test Diet	1000	20[b]	0.2	0.170 ± 0.0733	0.1 - 0.4	<0.0001[a]	0.0546
5	Heat-treated Control Diet	1000	27	0.7	0.685 ± 0.123	0.3 - 0.9	--	--
6	Positive Control Diet	0[c]	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 10,000 ng boric acid per mg diet wet weight. Not applicable (NA); there were no surviving *SCR*in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

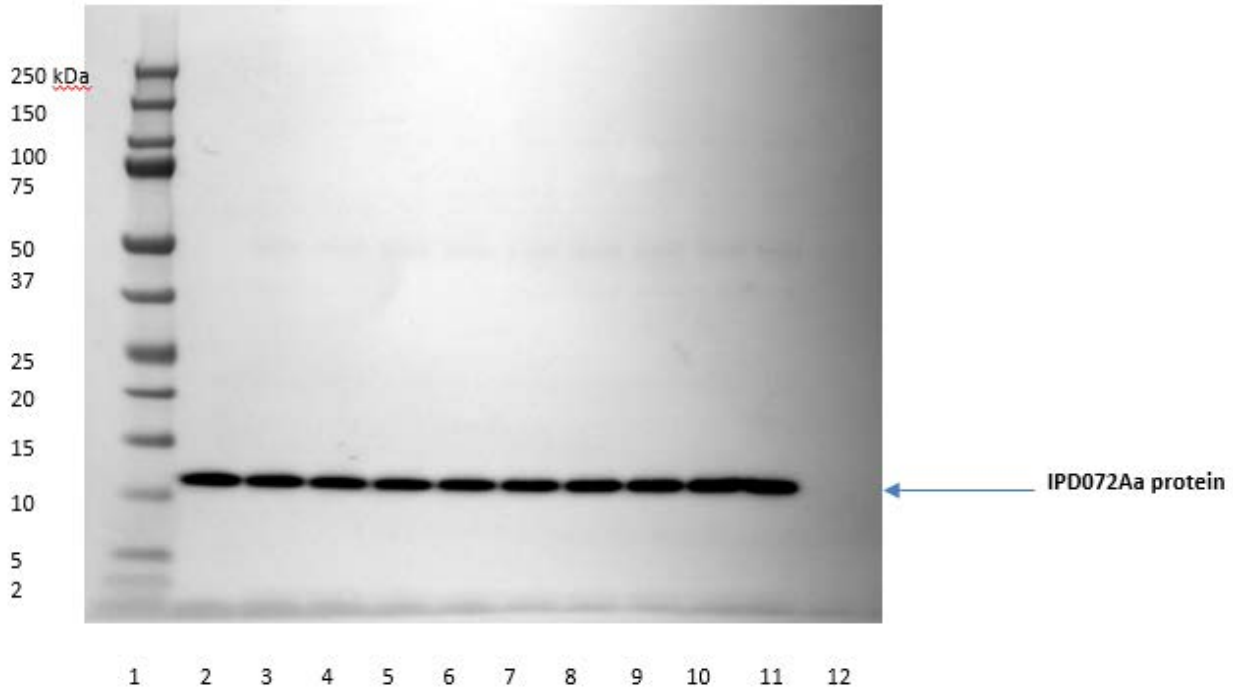
[b] One organism was lost during weighing; this organism was not included in the total number of surviving organisms for weight results.

[c] Treatment 6 contained a targeted concentration of 10,000 ng boric acid per mg diet wet weight.

Table 121. Summary of SCR IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	30	13.3	26	0.450 ± 0.148	0.1 - 0.9
B	Test Diet	100	30	93.3	2	0.100 ± 0	0.1 - 0.1

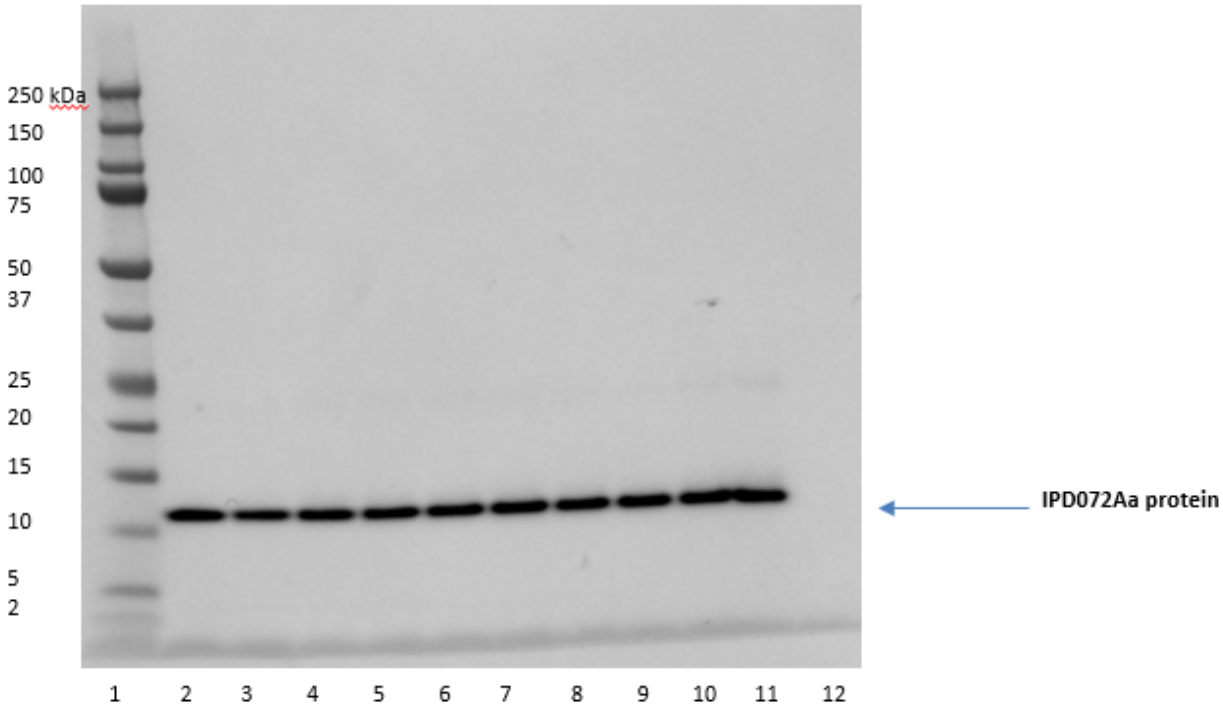
Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared from the same diet preparations used in Treatments 1 and 4, respectively, of the SCR bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet distribution process at which the samples were collected.

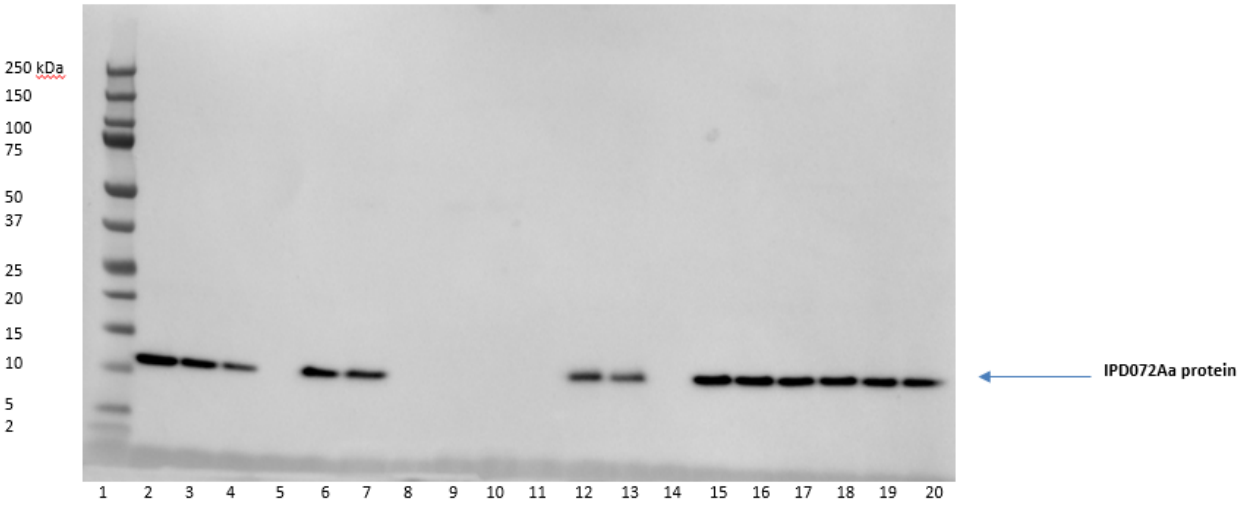
Figure 7. Homogeneity Assessment of the IPD072Aa Protein in SCR Bioassay Treatment 2, Day 0



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stages in the diet distribution process at which the samples were collected.

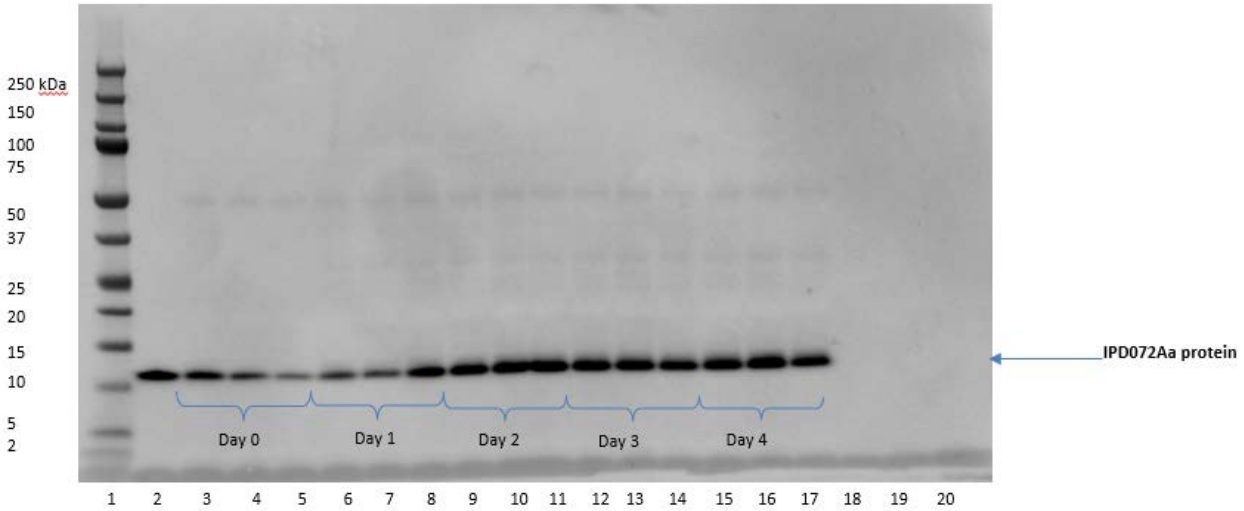
Figure 8. Homogeniety Assessment of the IPD072Aa Protein in Treatment 4, Day 4



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	1X LDS Sample Buffer Blank
2	Test Substance (20 ng)	12	Heat-Treated Control Diet (Treatment 5)
3	Test Substance (10 ng)	13	Heat-Treated Control Diet (Treatment 5)
4	Test Substance (5 ng)	14	1X LDS Sample Buffer Blank
5	1X LDS Sample Buffer Blank	15	Treatment 2 IPD072Aa Protein Test Dosing Solution (Day 0)
6	IPD072Aa Protein Test Diet (Treatment 4 dose confimation)	16	Treatment 2 IPD072Aa Protein Test Dosing Solution (after final use)
7	IPD072Aa Protein Test Diet (Treatment 2 dose confirmation)	17	Treatment 3 IPD072Aa Protein Test Dosing Solution (Day 0)
8	1X LDS Sample Buffer Blank	18	Treatment 3 IPD072Aa Protein Test Dosing Solution (after final use)
9	Bioassay Control Diet (Treatment 1)	19	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 0)
10	Bioassay Control Diet (Treatment 1)	20	Treatment 4 IPD072Aa Protein Test Dosing Solution (after final use)

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 samples were loaded at the same matrix concentration as Treatment 2. Treatment 2 and Treatment 4 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective test dosing solution.

Figure 9. Dose Confirmation of the IPD072Aa Protein in Treatments 2 and 4, Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5, and Stability of the IPD072Aa Protein in the Test Dosing Solutions



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
2	Test Substance (10 ng)	12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	18	1X LDS Sample Buffer Blank
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	19	1X LDS Sample Buffer Blank
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	20	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Days 0-4 refer to the number of days for which samples were stored under bioassay conditions prior to analysis.

Figure 10. Stability Assessment of the IPD072Aa Protein in Treatment 2 under Bioassay Conditions

F2.a. Southern Corn Rootworm IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the SCR bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in two test diets (Treatments 2 and 4; 100 ng and 1000 ng IPD072Aa protein per mg diet wet weight, respectively). Homogeneity assessment of two test diets on one day of diet preparation each is considered representative of both days of diet preparation for all test diets. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively), stability under bioassay conditions of the IPD072Aa protein in Treatment 2, and the stability of the IPD072Aa protein dosing solutions used to prepare Treatments 2, 3, and 4, were also assessed.

Preparation of Solutions

Bulk dosing solutions were prepared on Day 0 of the *SCR* bioassay and maintained chilled (in a 4 °C refrigerator unit or on wet ice) until use. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.140 mg/ml, 0.699 mg/ml, and 1.40 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution for the SCR bioassay, samples were collected as shown in Table 122.

Table 122. SCR IPD072Aa Protein Diet Collection Samples

Treatment	Diet Distribution Day	Number of Samples	Analysis
2	0	5 beginning	Homogeneity of IPD072Aa protein
		5 middle	
		5 end	
4	4	5 beginning	
		5 middle	
		5 end	
4	0	1	Visual confirmation of IPD072Aa protein dose
2	4	1	
1	0, 4	1 from each treatment each day	Verify presence or absence of immunodetectable IPD072Aa protein
5			
1	0	25[a]	Oven dry weight equivalence
2	0	23[b]	Stability under bioassay conditions

[a] Five samples were collected into tubes; the remaining samples were distributed to bioassay plates and placed under bioassay conditions.

[b] Three samples were collected into tubes; the remaining samples were distributed to bioassay plates and placed under bioassay conditions.

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

Samples were collected during one day of diet preparation for verification of stability of IPD072Aa protein in Treatment 2 under bioassay conditions. For the Day 0 assessment, five samples of Treatment 1 and three samples of Treatment 2 were collected into tubes and prepared for SDS-PAGE analysis. Extracts and remaining diet were stored frozen (-80 °C freezer unit) until analysis. For Days 1-4 assessment, samples of Treatment 1 and 2 (five each per day) were randomly distributed to bioassay plates and placed under bioassay conditions using the same methods and conditions used in the *SCR* bioassay, except they were not infested with larvae. For each day of stability analysis (Days 1-4), one bioassay plate for each of Treatment 1 and 2 (five samples each plate) was removed from bioassay conditions. Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1, 2, 4, and 5 were weighed to approximately 40 mg on wet ice in preparation for SDS-PAGE. For dose confirmation and assessment of homogeneity in Treatments 2 and 4, Day 0 stability under bioassay conditions in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X lithium dodecyl sulfate (LDS) sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing dithiothreitol, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance and sub-samples of the test dosing solutions (Day 0 and following final use) were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance or test dosing solution, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown in Table 123.

Table 123. SCR IPD072Aa Protein SDS-PAGE Samples

Gel	Treatment	Day(s)	Number of Samples
Dose confirmation of IPD072Aa protein	2	4	1
	4	0	1
Homogeneity	2	0	3 beginning
			3 middle
			3 end
	4	4	3 beginning
			3 middle
			3 end
Stability under bioassay conditions	2	0-4	3 per timepoint
Stability of IPD072Aa protein in test dosing solutions	Used to prepare Treatment 2	Day 0, after final use	1 per timepoint
	Used to prepare Treatment 3		1 per timepoint
	Used to prepare Treatment 4		1 per timepoint
Presence/absence of IPD072Aa protein	1	0, 4	1 per timepoint
	5		1 per timepoint

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature.

Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F2.b. Southern Corn Rootworm IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the *SCR*(*SCR*) bioassay was evaluated by conducting a 7-day bioassay using *Diabrotica virgifera virgifera* (*WCR*), a species sensitive to IPD072Aa protein. The *WCR* bioassay was initiated on Day 0 of the *SCR* bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

- Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)
- Treatment B: Test Diet (containing a portion of Treatment 4 and targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Samples were collected from Treatments 1 and 4 on Day 0 and Day 4 diet preparation for the SCR bioassay and were used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the SCR bioassay, resulting in a 10% incorporation of the SCR diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 4 from the SCR bioassay, resulting in a 10% incorporation of the SCR diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 121. Weight data were summarized as means, standard deviations, and ranges inTable 121.

F3. Evaluation of the Survival and Weight of Colorado Potato Beetle Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit

Carrier

The carrier for the Colorado Potato Beetle (CPB) bioassay consisted primarily of Stonefly Heliothis diet.

The carrier for the sensitive insect WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentrations in the test diets (Treatment 2, 3, and 4).

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section F3.a. Colorado Potato Beetle IPD072Aa Bioassay Analytical Phase.

Positive Control

The positive control substance used to prepare Treatment 6 consisted of cryolite (AlF_6Na_3).

Test System

The test system was *Leptinotarsa decemlineata* (Colorado potato beetle; Coleoptera: Chrysomelidae). CPB was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. CPB eggs were obtained from French Agricultural Research, Inc. (Lamberton, MN, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was *Diabrotica virgifera virgifera* (western corn rootworm; Coleoptera: Chrysomelidae). The test system was chosen because *WCR* is an insect

sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

CPB larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 5000 ng cryolite per mg carrier dry weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a 12-well bioassay plate and contained two replicates from each treatment. Each treatment was fed to a target of 30 *CPB* individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Days 2, 4, and 6 and missing and dead organisms were recorded. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatment 1 and Treatment 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the CPB bioassay.

Bias in the CPB bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing and appropriate assay controls.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Bulk dosing solutions for Treatments 1, 2, 3, 4, and 5 were prepared and maintained as described in Appendix A. On each day of diet preparation, each dosing solution was mixed with carrier in a 2.8:1 ratio (*i.e.*, 2.8 ml of dosing solution to 1 g of carrier) to generate Treatments 1-5. Treatment 6 was prepared by mixing cryolite with carrier to a nominal concentration of 5000 ppm by dry weight and then combining with ultrapure water in a 2.8:1 ratio.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1, 2, 3, 4, and 5 were collected for characterization of diets as described in section F3.a. Colorado Potato Beetle IPD072Aa Bioassay Analytical Phase. The positive control diet (Treatment 6) was not characterized.

CPB Bioassay

A bioassay was conducted to determine the response of CPB to IPD072Aa protein exposure via oral ingestion. CPB eggs were incubated in an environmental chamber until the eggs hatched. CPB neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One CPB neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 7 days. On Days 2, 4, and 6, new bioassay plates were prepared with fresh diet as described for Day 0, living CPB larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparisons were made between CPB fed diet containing IPD072Aa (Treatment 2, 3, and 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

CPB fed an artificial insect diet containing IPD072Aa protein (m_T) (Treatments 2, 3, and 4) was greater than the mortality rate of those fed the bioassay control diet (m_C) (Treatment 1). The corresponding hypothesis tests were

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test

Weight

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight was treated as the response and analyzed using model:

$$y_{ij} = W_i + \varepsilon_{ij}$$

where W_i denotes the mean of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} sample of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_{\varepsilon})$ were independently and identically distributed. This assumption was confirmed by inspection of the residuals from the fitted model.

To test if the weight of *Leptinotarsa decemlineata* fed an artificial insect diet containing IPD072Aa protein (w_T ; Treatments 2, 3, and 4) was less than the weight of those fed the bioassay control diet (w_C ; Treatment 1), the corresponding hypothesis tests were

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis, and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Demonstration of IPD072Aa Protein Activity in the *CPB* Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 4 in the CPB bioassay. The initial sensitive insect bioassay did not meet the acceptability criteria; therefore, it was repeated. Details regarding the second run of the sensitive insect bioassay are provided in the Sensitive Insect Assay section below.

Results and Discussion

The CPB bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 6) group exceeded 80%, as shown inTable 124.

The mortality of CPB fed the test diets containing 100 ng/mg, 500 ng/mg, and 1000 ng/mg IPD072Aa protein (Treatments 2, 3, and 4, respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1), as shown in Table 124. Mortality was 0% in Treatment 2 (P-value 1.0000), 6.67% in Treatment 3 (2 dead; P-value 0.2458), and 3.33% in Treatment 4 (1 dead; P-value 0.5000). Mortality was 0% in Treatment 1.

The mean weight of CPB fed Treatments 2, 3, and 4 was not significantly less than those fed the bioassay control diet (Treatment 1), as shown in Table 125. Mean weight was 20.8 mg in Treatment 2 (P-value 0.1105), 25.9 mg in Treatment 3 (P-value 0.6645), and 30.3 mg in Treatment 4 (P-value 0.9683). Mean weight was 24.6 mg in Treatment 1.

Western blot analysis visually confirmed the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 (Figure 11; Figure 12; Figure 13; Figure 14) and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2 (Figure 15). Homogeneity assessment of the three test diets on one day of diet preparation each is considered representative of all days of diet preparation for all test diets. The stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 (Figure 16) and the presence or absence of immunodetectable IPD072Aa protein in Treatment 1 and Treatment 5 (Figure 17) were also assessed. The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 17); however, the band was less intense than that of the test diet (Treatment 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 17).

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 126. Summary of CPB IPD072Aa Protein Sensitive Insect Bioassay Results. The initial WCR bioassay failed to meet the acceptability criteria and the bioassay was repeated. Details regarding the second run of the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section below. The second run met the acceptability criterion as the combined dead and missing larval count did not exceed 30% in the bioassay control diet (Treatment A) group. The biological activity of the IPD072Aa protein in the stored test dosing solution used to prepare Treatment 4 for the CPB bioassay was demonstrated by increased mortality of WCR fed Treatment B. The stability of the IPD072Aa protein in the test dosing solution used to prepare Treatment 4 in the CPB bioassay and Treatment B in the WCR sensitive insect bioassay was visually confirmed using Western blot analysis (Figure 18).

Conclusion

The results demonstrated exposure to a concentration of 100, 500, or 1000 ng IPD072Aa protein per mg diet (Treatments 2, 3, and 4, respectively) had no adverse effect on survival and weight of CPB.

Table 124. Summary Analysis of CPB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	0	0	1.0000
3	Test Diet	500	30	2	6.67	0.2458
4	Test Diet	1000	30	1	3.33	0.5000
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0	29[a]	29	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 5000 ng cryolite per mg carrier dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 125. Summary Analysis of CPB IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	24.6 (20.3 - 28.8)	0.8 - 48.8	--
2	Test Diet	100	30	20.8 (16.6 - 25.1)	1.0 – 59.0	0.1105
3	Test Diet	500	28	25.9 (21.5 - 30.2)	2.5 - 39.8	0.6645
4	Test Diet	1000	29	30.3 (26.0 - 34.6)	3.6 – 49.0	0.9683
5	Heat-treated Control Diet	1000	29	32.2 ± 14.7[a]	4.9 - 64.2	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 5000 ng cryolite per mg carrier dry weight. Not applicable (NA); there were no surviving *CPB* in the positive control diet group.

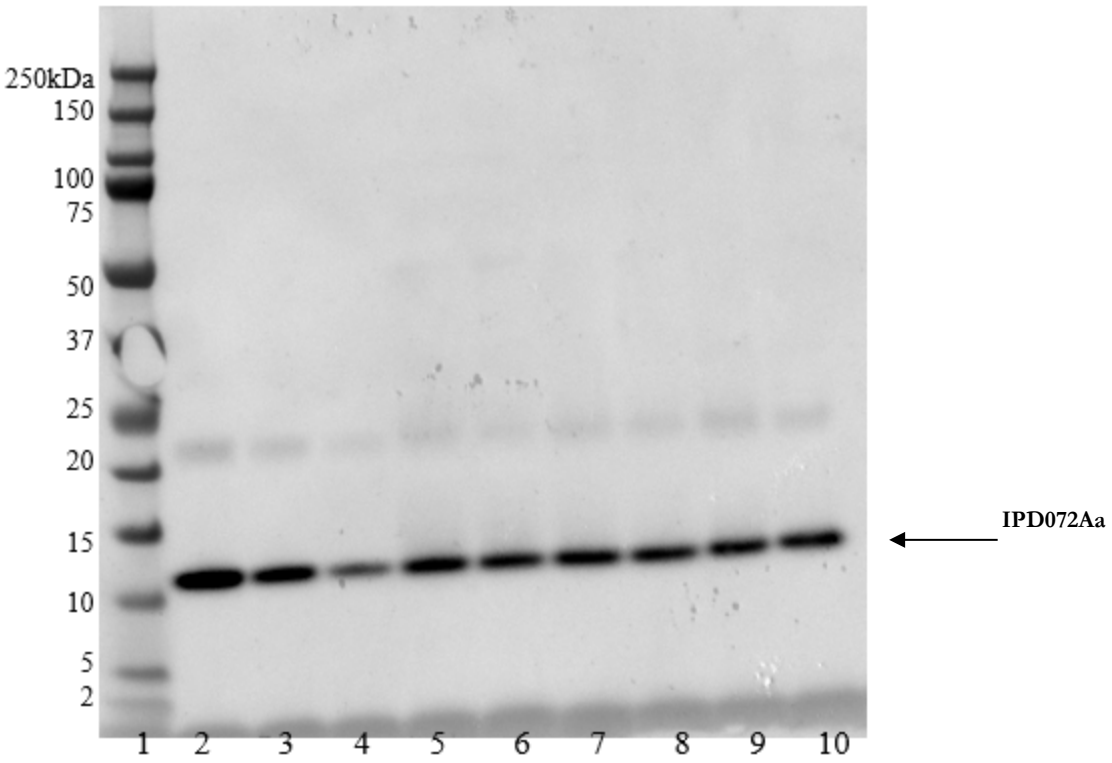
[a]Standard deviation is provided for mean values not subjected to a *t*-test.

Table 126. Summary of CPB IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0	29[a]	7	24.1
B	Test Diet	100	27[a]	27	100

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared using the same control dosing solution and IPD072Aa protein test dosing solution, respectively, used to prepare Treatments 1 and 4 for the *CPB* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

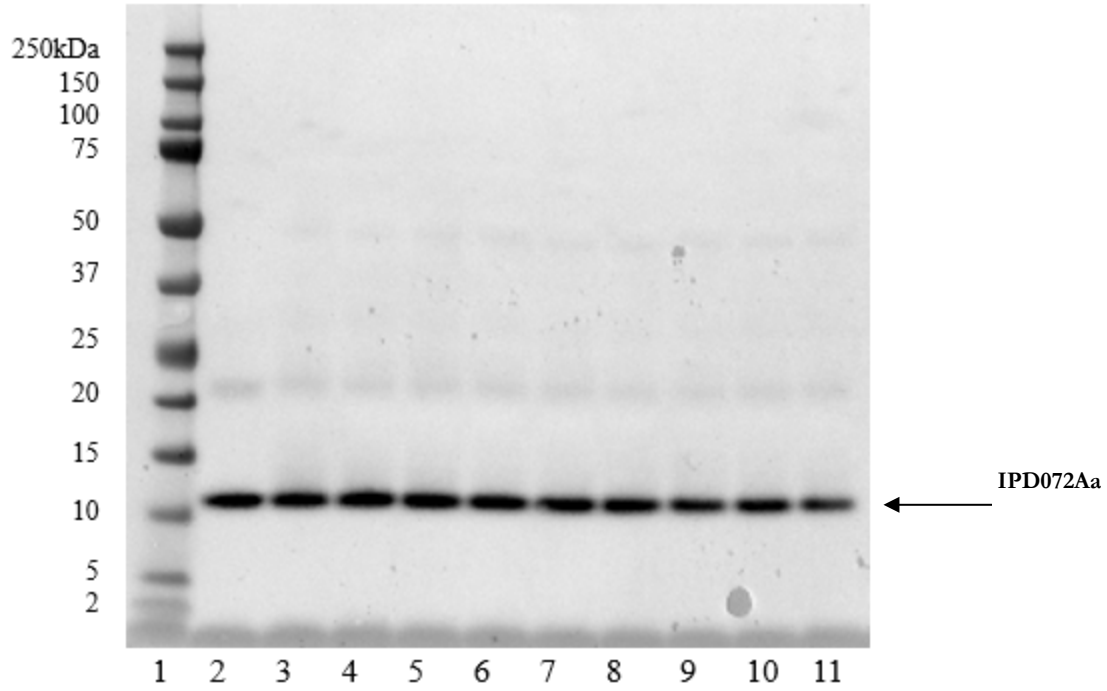
[a]Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	Day 0 IPD072Aa Protein Test Diet (Treatment 2; Rep 1; 10 ng)
6	Day 0 IPD072Aa Protein Test Diet (Treatment 2; Rep 2; 10 ng)
7	Day 2 IPD072Aa Protein Test Diet (Treatment 3; Rep 1; 10 ng)
8	Day 2 IPD072Aa Protein Test Diet (Treatment 3; Rep 2; 10 ng)
9	Day 6 IPD072Aa Protein Test Diet (Treatment 4; Rep 1; 10 ng)
10	Day 6 IPD072Aa Protein Test Diet (Treatment 4; Rep 2; 10 ng)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. The test diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment.

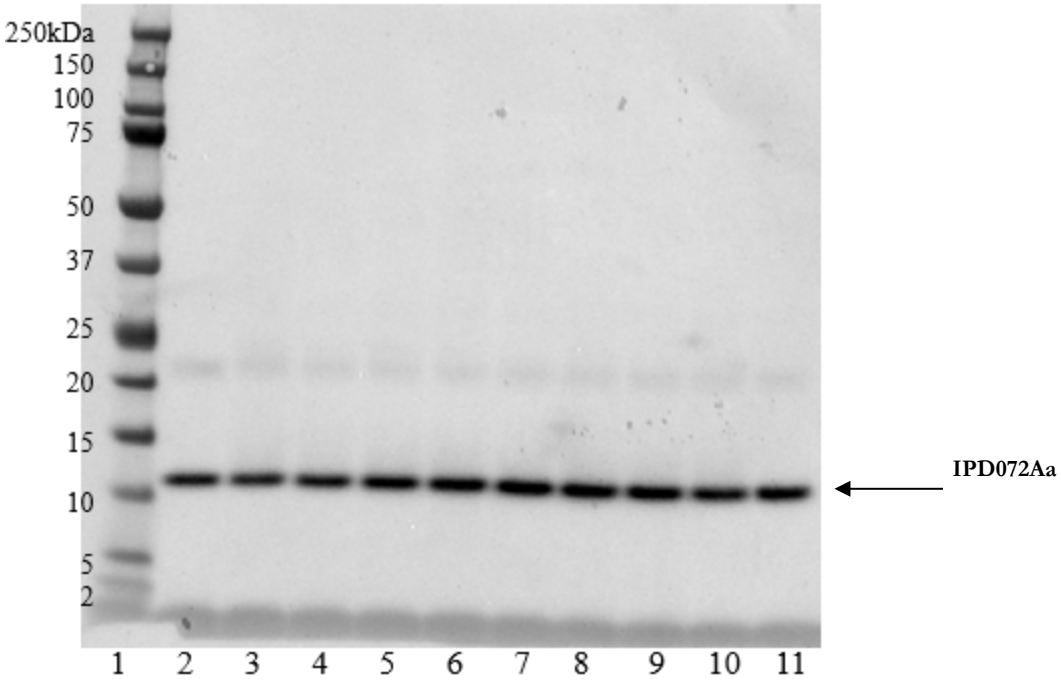
Figure 11. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3, and 4 on Days 0, 2, and 6



La2017ne	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet distribution process at which samples were collected.

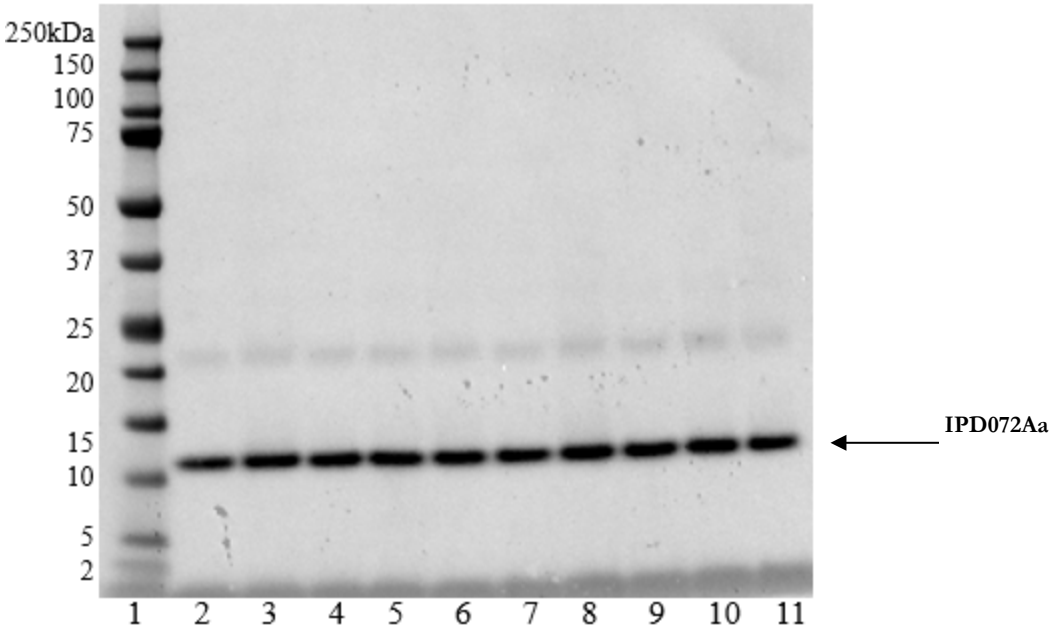
Figure 12. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2 on Day 0



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; End)
10	IPD072Aa Protein Test Diet (Treatment 3; End)
11	IPD072Aa Protein Test Diet (Treatment 3; End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 3. Beginning, middle, and end refer to the stages in the diet distribution process at which samples were collected.

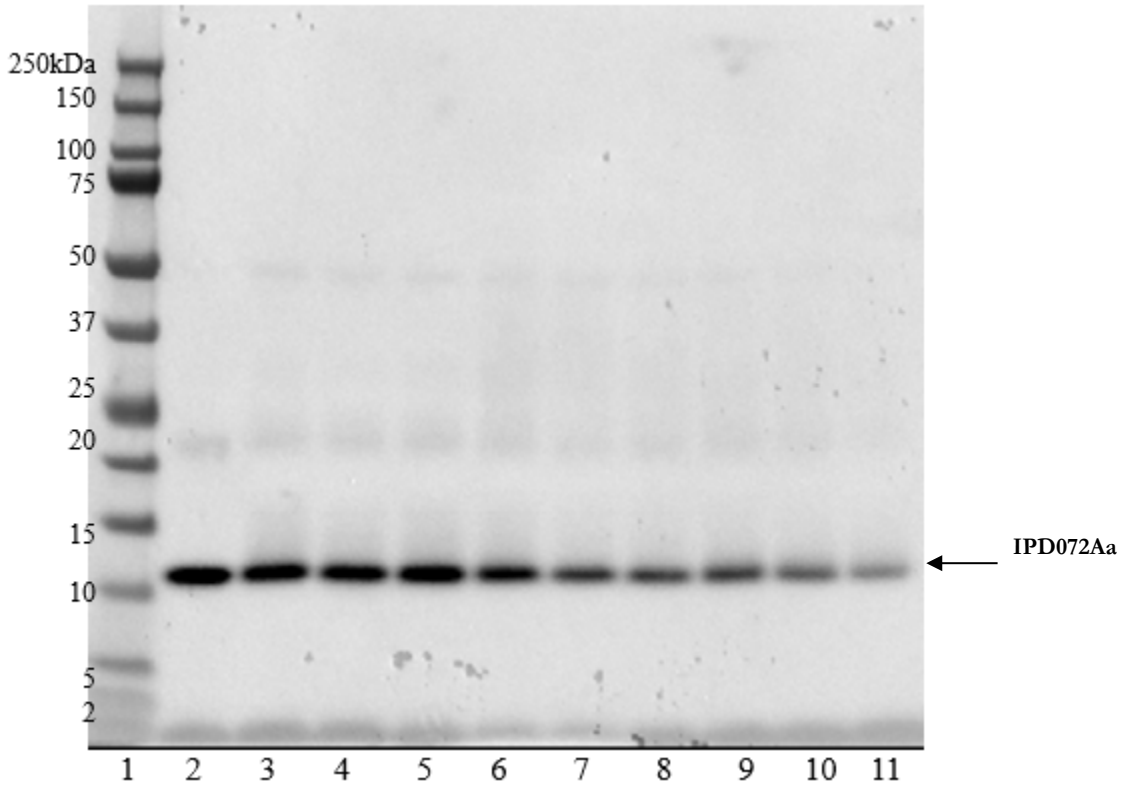
Figure 13. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3 on Day 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stages in the diet distribution process at which samples were collected.

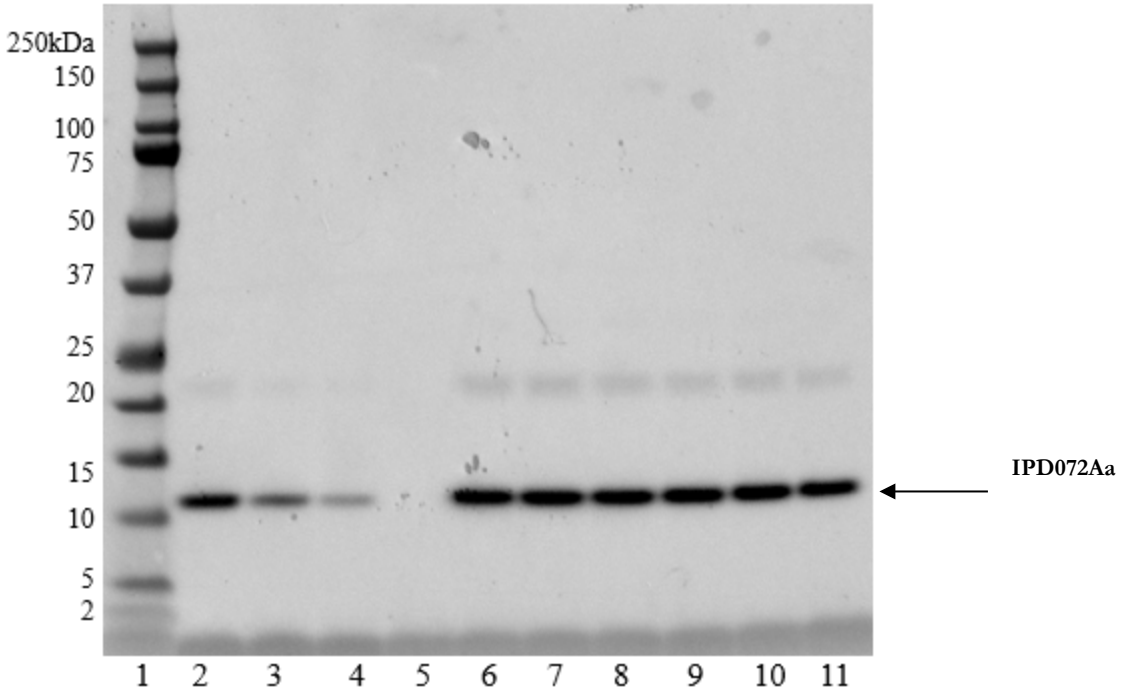
Figure 14. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4 on Day 6



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Days 0-2 refer to the number of days that samples were stored under bioassay conditions prior to analysis. On Day 1 and Day 2 of stability analysis, the IPD072Aa protein bands appeared slightly less intense than on Day 0; however, organisms were refed every other day.

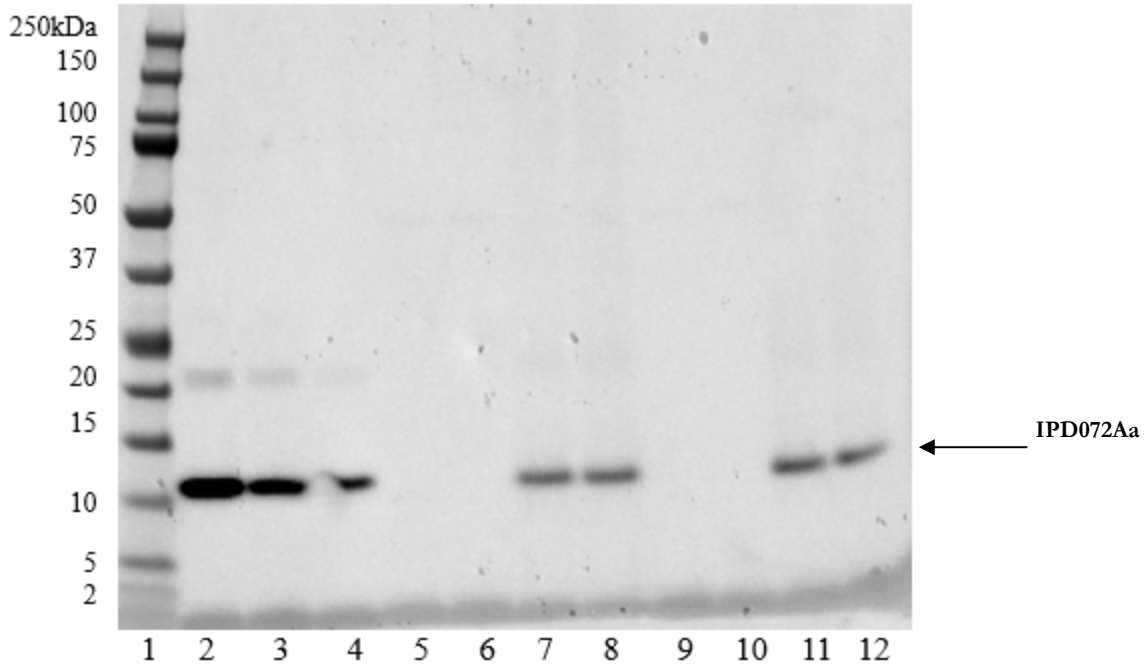
Figure 15. Stability Assessment of the IPD072Aa Protein in Treatment 2 under Bioassay Conditions



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 0)
7	Treatment 3 IPD072Aa Protein Test Dosing Solution (Day 0)
8	Treatment 2 IPD072Aa Protein Test Dosing Solution (Day 0))
9	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 6)
10	Treatment 3 IPD072Aa Protein Test Dosing Solution (Day 6)
11	Treatment 2 IPD072Aa Protein Test Dosing Solution (Day 6))

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. The test dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective test dosing solution.

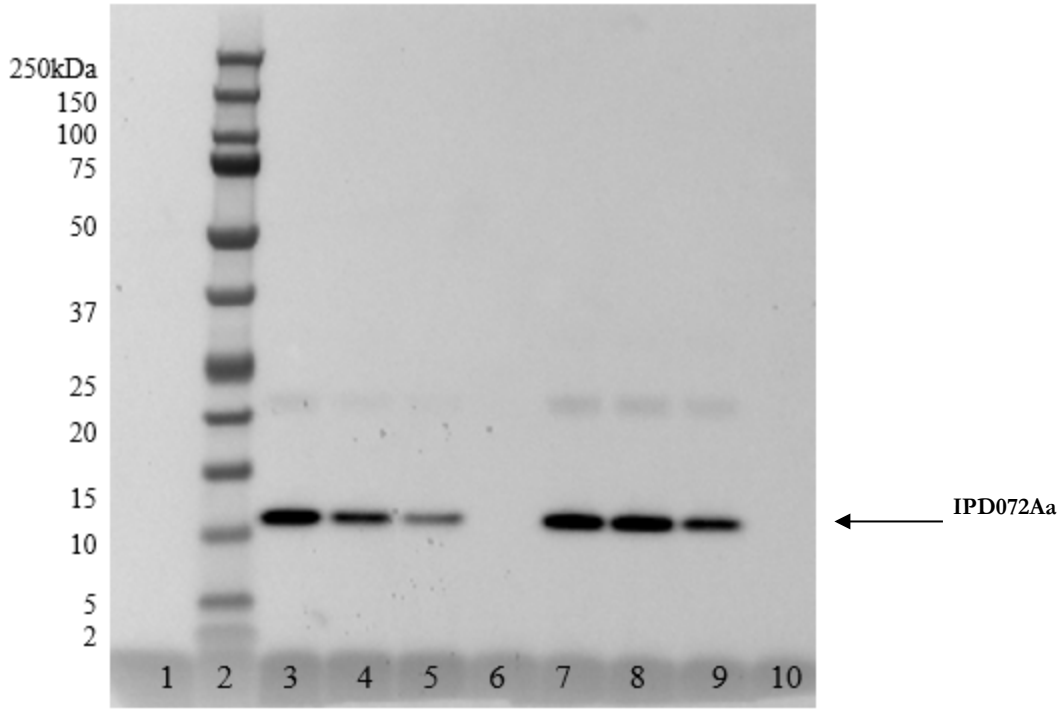
Figure 16. Stability Assessment of the IPD072Aa Protein in the Test Dosing Solutions Used to Prepare Treatments 2, 3, and 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	Bioassay Control Diet (Treatment 1, Day 0)
6	Bioassay Control Diet (Treatment 1, Day 0)
7	Heat-Treated Control Diet (Treatment 5, Day 0)
8	Heat-Treated Control Diet (Treatment 5, Day 0)
9	Bioassay Control Diet (Treatment 1, Day 6)
10	Bioassay Control Diet (Treatment 1, Day 6)
11	Heat-Treated Control Diet (Treatment 5, Day 6)
12	Heat-Treated Control Diet (Treatment 5, Day 6)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 and 5 samples were run in duplicate. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

Figure 17. Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatment 1 and Treatment 5, Day 0 and Day 6



Lane	Sample Identification
1	1X LDS Sample Buffer Blank
2	Pre-stained Protein Molecular Weight Marker
3	Test Substance (20 ng)
4	Test Substance (10 ng)
5	Test Substance (5 ng)
6	1X LDS Sample Buffer Blank
7	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 0)
8	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 6)
9	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 20 – after final use)
10	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test dosing solution used to prepare Treatment 4 for the *CPB* bioassay. The stored dosing solution was also used to prepare Treatment B for the *WCR* bioassay.

Figure 18. Further Assessment of the Stability of the IPD072Aa Protein in the Test Dosing Solution Used to Prepare Treatment 4

F3.a. Colorado Potato Beetle IPD072Aa Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the CPB bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets.

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of IPD072Aa protein in three test diets (Treatments 2, 3, and 4; 100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet wet weight, respectively). Homogeneity assessment of the three test diets on one day of diet preparation each is considered representative of all days of diet preparation for all test diets. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively), the stability under bioassay conditions of the IPD072Aa protein in Treatment 2, and the stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 for the CPB bioassay and Treatment B for the WCR bioassay were also assessed.

Preparation of Solutions

Bulk dosing solutions were prepared on Day 0 of the CPB bioassay and maintained chilled (in a refrigerator set at 4 °C or on wet ice) until use. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.136 mg/ml, 0.678 mg/ml, and 1.36 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution for the CPB bioassay, samples were collected as shown in **Table 127**

Table 127. CPB IPD072Aa Protein Diet Sample Collection

Treatment	Diet Preparation Day	Number of Samples	Analysis
2	0	5 beginning	Homogeneity of IPD072Aa protein
		5 middle	
		5 end	
3	2	5 beginning	
		5 middle	
		5 end	
4	6	5 beginning	
		5 middle	
		5 end	
2	0	NA (homogeneity sample for each treatment was used)	Visual confirmation of IPD072Aa protein dose
3	2		
4	6		
1	0, 6	1 from each treatment	Verify presence or absence of immunodetectable IPD072Aa protein
5			
1	0	5 (for Day 0 assessment)	Oven dry weight equivalence
		10 (distributed to bioassay plates)	
2	0	NA (Day 0 homogeneity samples were used)	Stability under bioassay conditions
2	0	10 (distributed to bioassay plates)	

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, the samples of Treatment 1 and Treatment 2 (10 each) distributed to bioassay plates were placed under bioassay conditions using the same methods and conditions used in the CPB bioassay, except they were not infested with larvae. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Day 1 and Day 2), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for

SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-2) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and SDS-PAGE

Sub-samples of Treatments 1, 2, 3, 4, and 5 were weighed to approximately 40 mg on wet ice in preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For dose confirmation and assessment of homogeneity in Treatments 2, 3, and 4, Day 0 stability under bioassay conditions in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-2), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance and sub-samples of the test dosing solutions (Day 0 and following final use) were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance or test dosing solution, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 5 minutes. The following samples were then loaded into 4-12% Bis-Tris gels as shown in Table 128

Table 128. CPB IPD072Aa SDS-PAGE Samples

Gel	Treatment	Day(s)	Number of Samples
Dose confirmation of IPD072Aa protein	2	0	2
	3	2	2
	4	6	2
Homogeneity	2	0	3 beginning
			3 middle
			3 end
	3	2	3 beginning
			3 middle
			3 end
	4	6	3 beginning
			3 middle
			3 end
Stability under bioassay conditions	2	0-2	3 per timepoint
Stability of IPD072Aa protein in test dosing solutions	Used to prepare Treatment 2	Day 0, Day 6	1 per timepoint
	Used to prepare Treatment 3		1 per timepoint
	Used to prepare Treatment 4		1 per timepoint
Presence/absence of IPD072Aa protein	1	0, 3	1 per timepoint
	5		1 per timepoint
Re-assessment of stability of IPD072Aa protein in test dosing solution[a]	Used to prepare Treatment 4	0, 6, 20	1 per timepoint

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before and after the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST four times for at least 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F3.b. Colorado Potato Beetle IPD072Aa Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in the test dosing solution used to prepare Treatment 4 for the CPB bioassay was evaluated by conducting a 7-day bioassay using WCR (a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after completion of the CPB bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 10% of a representative Treatment 1 consisting of the refrigerated bioassay control dosing solution and carrier used in the CPB bioassay by wet weight of the WCR diet)

Treatment B: Test Diet (containing 10% of a representative Treatment 4 consisting of the refrigerated test control dosing solution and carrier used to prepare Treatment 4 of the *CPB* bioassay; targeting 100 ng IPD072Aa protein per mg *WCR* diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 *WCR* individuals.

Diet Preparation

Refrigerated bioassay control dosing solution and IPD072Aa protein test dosing solution prepared for the CPB bioassay were mixed with a carrier consisting primarily of Stonefly Heliothis diet in a 2.8:1 ratio (i.e. 2.8 ml dosing solution to 1 g carrier) to prepare representative Treatments 1 and 4, respectively, using the same procedures used to prepare diets for the CPB bioassay. The representative Treatments 1 and 4 were used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For each respective treatment, ultrapure (defined as American Society for Testing and Materials ASTM Type 1) water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with the prepared representative Treatment 1, resulting in a 10% incorporation of the CPB diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with the prepared representative Treatment 4, resulting in a 10% incorporation of the CPB diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one

organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 126.

F4. Evaluation of the Survival and Weight of Mealworm Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the MWM bioassay consisted primarily of Stonefly Heliothis diet.

The carrier for the sensitive insect (WCR) bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section F4.a. Mealworm IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was *Tenebrio molitor* (mealworm; Coleoptera: Tenebrionidae; MWM). MWM was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. MWM eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was WCR. The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

MWM larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a 12-well bioassay plate and contained two replicates from each treatment. Each treatment was fed to a target of 30 MWM individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every three to four days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed. Stability of IPD072Aa protein in the carrier under bioassay conditions was confirmed under a separate Pioneer study and was not reassessed during this study. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the MWM bioassay.

Bias in the MWM bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation

Bulk dosing solutions for Treatments 1-5 were prepared and maintained as described in section F4.a. Mealworm IPD072Aa Protein Bioassay Analytical Phase. The boric acid dosing solution for Treatment 6 was prepared by solubilizing boric acid then diluting individual aliquots in ultrapure water to achieve a nominal concentration of 12,500 ng/mg diet wet weight. Aliquots were maintained chilled (in a refrigerator set at 4 °C or on wet ice) until use. On each day of diet preparation, each dosing solution was mixed with carrier in a 3:1 ratio (*i.e.*, 3 ml of dosing solution to 1 g of carrier), generating Treatments 1-6.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1-5 were collected for characterization of diets as described in section F4.a. Mealworm IPD072Aa Protein Bioassay Analytical Phase. The positive control diet (Treatment 6) was not characterized. A portion each of Treatments 1 and 4 from Day 0 and Day 4 diet preparation was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay as section F4.b. Mealworm IPD072Aa Protein Sensitive Insect Bioassay.

MWM Bioassay

A bioassay was conducted to determine the response of MWM to IPD072Aa protein exposure via oral ingestion. MWM eggs were incubated in an environmental chamber until the eggs hatched. MWM neonates were used in the bioassay within 36 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One MWM neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 14 days. Every three to four days, new bioassay plates were prepared with fresh diet as described for Day 0, living MWM larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparisons were made between MWM fed diet containing IPD072Aa protein (Treatments 2, 3, and 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

MWM fed an artificial insect diet containing IPD072Aa protein (m_T) (Treatments 2, 3, and 4) was greater than the mortality rate of those fed the bioassay control diet (m_C) (Treatment 1). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight was treated as the response and analyzed using the model:

$$y_{ij} = W_i + \varepsilon_{ij}$$

where W_i denotes the mean of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} sample of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$ were independently and identically distributed. This assumption was confirmed by inspection of the residuals from the fitted model.

To test if the weight of MWM fed an artificial insect diet containing IPD072Aa protein (w_T) (Treatments 2, 3, and 4) was less than the weight of those fed the bioassay control diet (w_C) (Treatment 1), the corresponding hypothesis tests were

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis, and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Demonstration of IPD072Aa Protein Activity in the MWM Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of IPD072Aa protein in Treatment 4 in the MWM bioassay. Details regarding the sensitive insect bioassay are provided in section F4.b. Mealworm IPD072Aa Protein Sensitive Insect Bioassay.

Results and Discussion

The MWM bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 6) group exceeded 80%, as shown in Table 129.

The mortality of MWM fed the test diets containing 100 and 500 ng/mg IPD072Aa protein (Treatments 2 and 3; 6 and 10 dead, 20.0% and 33.3% mortality, P-value = 0.3653 and 0.0626, respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1; 4 dead; 13.3% mortality), as shown in Table 129. The mortality of MWM fed the test diet containing 1000 ng/mg IPD072Aa protein (Treatment 4; 15 dead, 50% mortality; P-value = 0.0024) was significantly greater than those fed Treatment 1, as shown in Table 129.

The mean weight of MWM fed Treatment 2 (0.913 mg; P-value 0.7792) was not significantly less than those fed Treatment 1 (0.858 mg), as shown in Table 2. The mean weights of MWM fed Treatments 3 and Treatment 4 (0.665 mg and 0.580 mg; P-values = 0.0057 and 0.0005, respectively) were significantly less than those fed Treatment 1, as shown in Table 130.

Western blot analysis visually confirmed the homogeneity and the dose of the IPD072Aa protein in Treatments 2, 3, and 4 Figure 19, Figure 20, Figure 21, and Figure 22. Homogeneity assessment of each of the three test diets on one day of diet preparation each is considered representative of all days of diet preparation for each test diet. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed (Figure 23 and Figure 24). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 23); however, the band was less intense than the band for the test substance at the same concentration (Lane 3; Figure 23). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 23).

Observed larval mortality and weight data for the WCR sensitive insect bioassay is summarized in Table 129 and Table 130. The WCR bioassay met the acceptability criterion (F4.b. Mealworm IPD072Aa Protein Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in Treatment 4 of the MWM bioassay was demonstrated by increased mortality and decreased

weight in WCR fed the test diet (Treatment B; described in section F4.b. Mealworm IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated the mortality for MWM fed the test diets containing 100 ng IPD072Aa protein per mg diet (Treatment 2; 20.0%) and 500 ng IPD072Aa protein per mg diet (Treatment 3; 33.3%) was not significantly greater than the mortality for MWM fed the bioassay control diet (Treatment 1; 13.3%). A statistically significant difference was observed in mortality between MWM fed the test diet containing 1000 ng IPD072Aa protein per mg diet (Treatment 4; 50.0%) and those fed Treatment 1. The mean weight for MWM fed Treatment 2 (0.913 mg) was not significantly less than the mean weight for MWM fed Treatment 1 (0.858 mg). Statistically significant differences were observed in mean weight between MWM fed Treatments 3 and 4 (0.665 mg and 0.580 mg, respectively) and those fed Treatment 1.

Table 129. Summary Analysis of MWM IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	4	13.3	--
2	Test Diet	100	30	6	20.0	0.3653
3	Test Diet	500	30	10	33.3	0.0626
4	Test Diet	1000	30	15	50.0	0.0024[a]
5	Heat-treated Control Diet	1000	30	4	13.3	--
6	Positive Control Diet	0	30	29	96.7	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight. Treatment 6 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.
[a] A statistically significant difference (P-value < 0.05) was observed.

Table 130. Summary Analysis of MWM IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	26	0.858 (0.760 - 0.955)	0.4 - 1.5	--
2	Test Diet	100	24	0.913 (0.811 - 1.01)	0.5 - 1.5	0.7792
3	Test Diet	500	20	0.665 (0.554 - 0.776)	0.3 - 1.1	0.0057[a]
4	Test Diet	1000	15	0.580 (0.451 - 0.709)	0.4 - 1.1	0.0005[a]
5	Heat-treated Control Diet	1000	26	0.977 ± 0.208[b]	0.4 - 1.2	--
6	Positive Control Diet	0	1	0.400[c]	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 12,500 ng boric acid per mg diet wet weight. Not applicable (NA); there was only one surviving *MWM* in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Standard deviation is provided for mean values not subjected to a *t*-test.

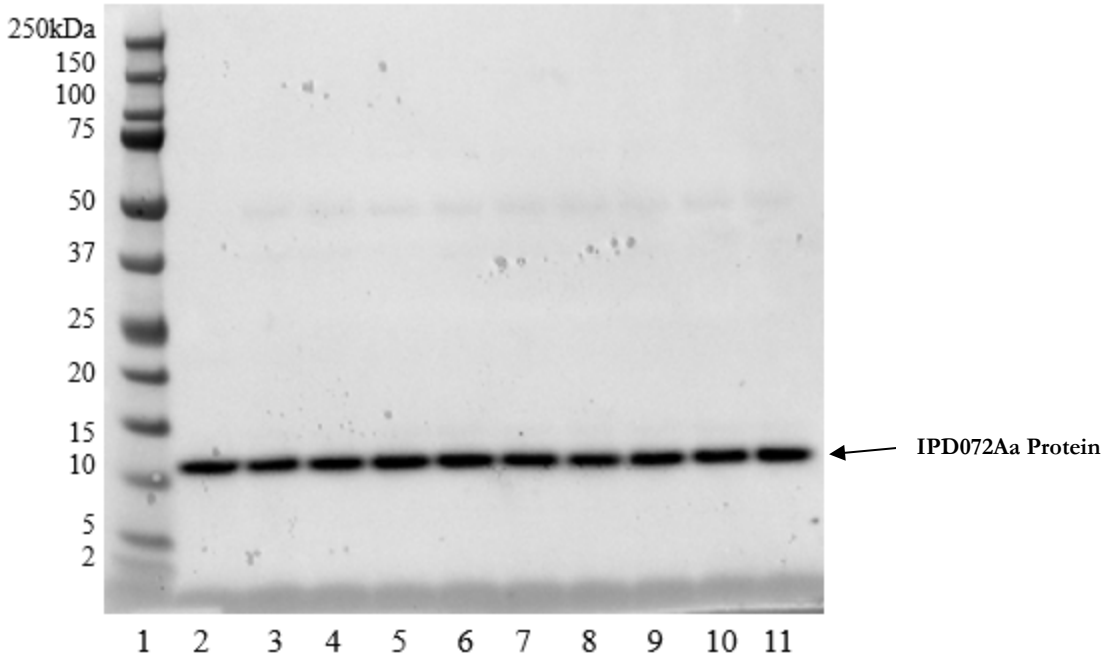
[c]The reported mean is the weight value of the one surviving larva.

Table 131. Summary of MWM IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Survived Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	30	0	30	0.383 ± 0.146	0.1 - 0.6
B	Test Diet	100	28[a]	85.7	4	0.100 ± 0.00	0.1 - 0.1

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera)* bioassay were prepared from the same diet preparations used in Treatments 1 and 4, respectively, of the *MWM* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

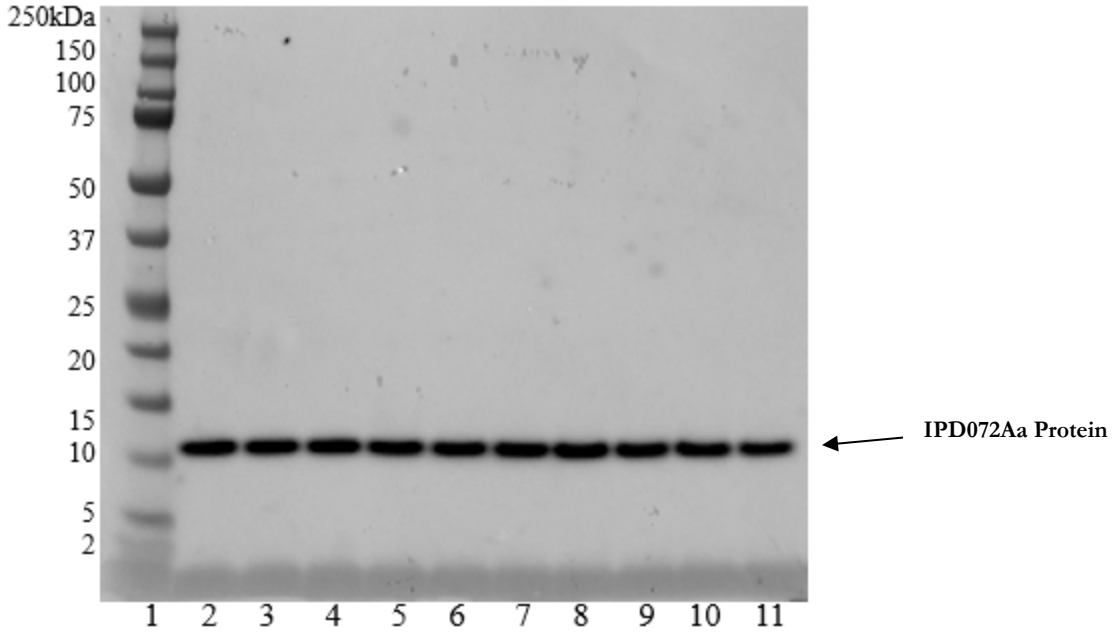
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 0; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 0; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 0; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; Day 0; End)
10	IPD072Aa Protein Test Diet (Treatment 2; Day 0; End)
11	IPD072Aa Protein Test Diet (Treatment 2; Day 0; End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

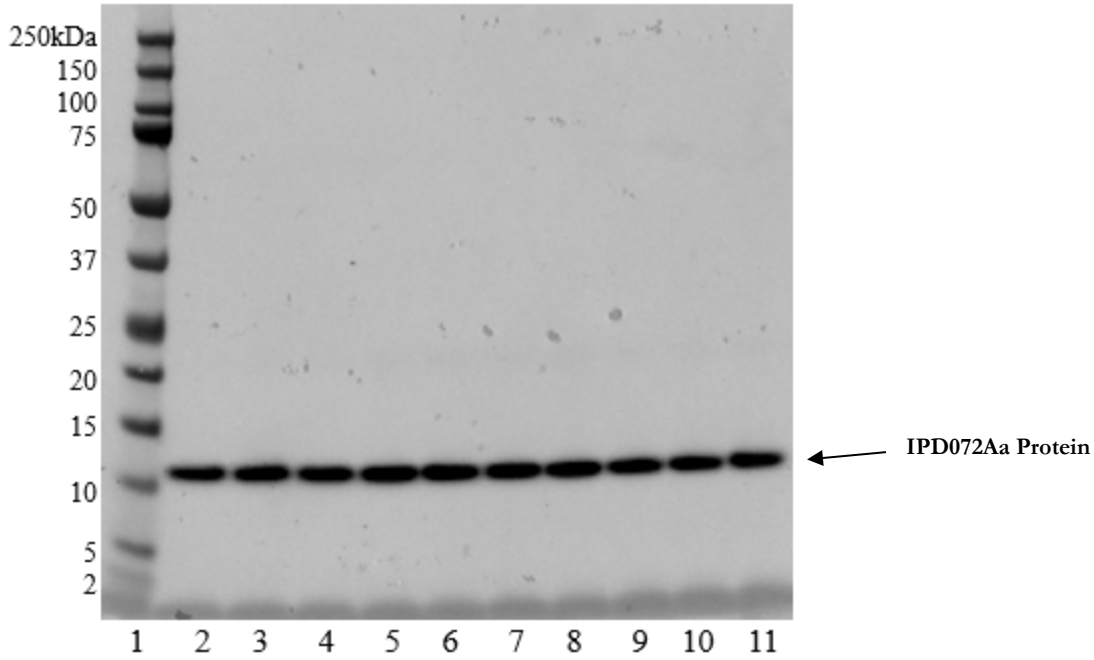
Figure 19. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2, Day 0



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Day 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Day 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Day 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Day 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Day 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Day 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; Day 4; End)
10	IPD072Aa Protein Test Diet (Treatment 3; Day 4; End)
11	IPD072Aa Protein Test Diet (Treatment 3; Day 4; End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 3. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

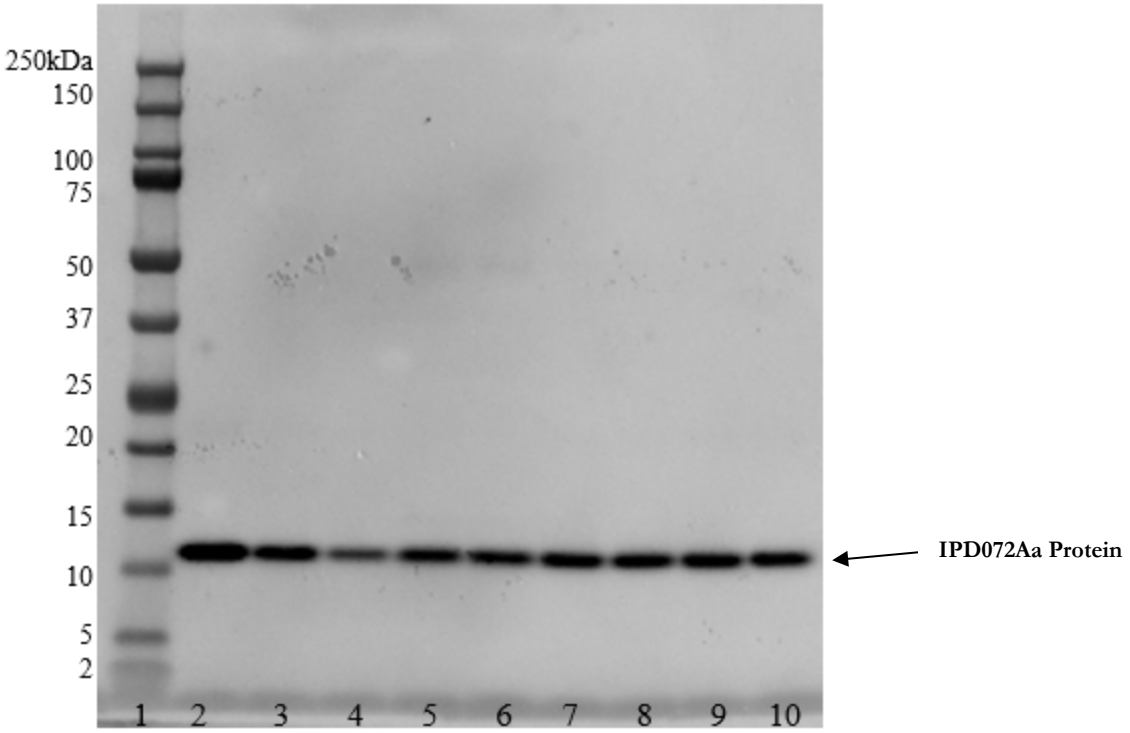
Figure 20. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3, Day 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Day 11; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Day 11; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Day 11; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Day 11; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Day 11; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Day 11; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; Day 11; End)
10	IPD072Aa Protein Test Diet (Treatment 4; Day 11; End)
11	IPD072Aa Protein Test Diet (Treatment 4; Day 11; End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

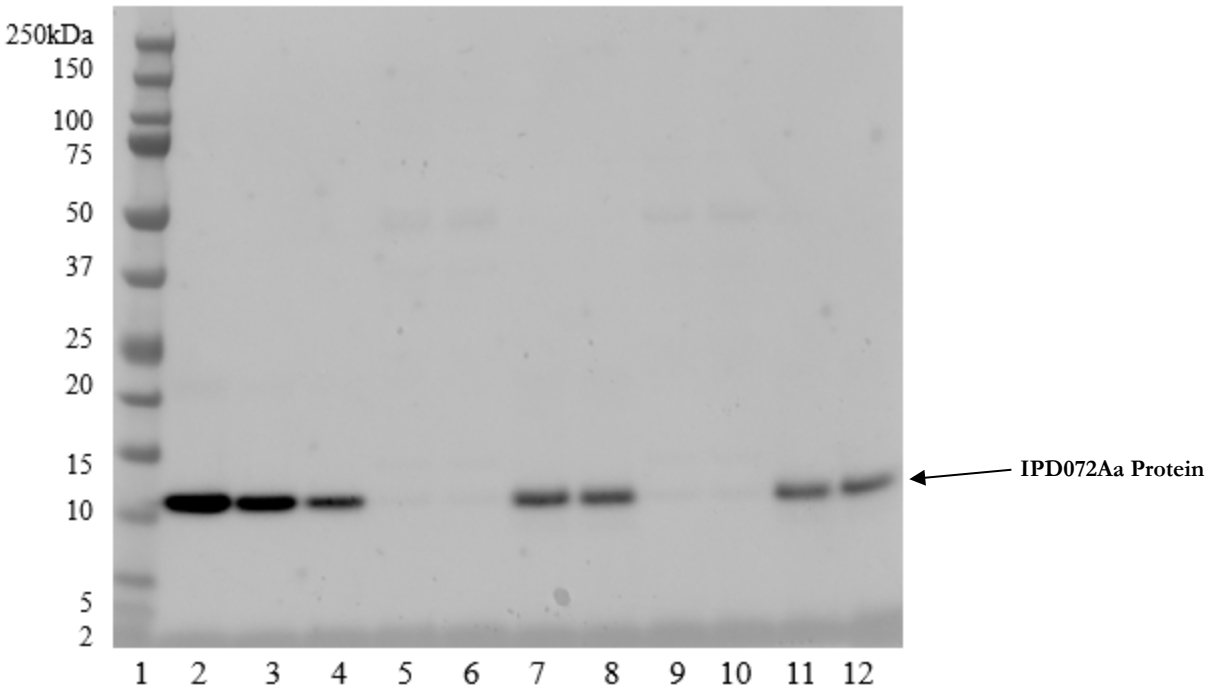
Figure 21. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4, Day 11



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
7	IPD072Aa Protein Test Diet (Treatment 3; Day 4)
8	IPD072Aa Protein Test Diet (Treatment 3; Day 4)
9	IPD072Aa Protein Test Diet (Treatment 4; Day 11)
10	IPD072Aa Protein Test Diet (Treatment 4; Day 11)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment.

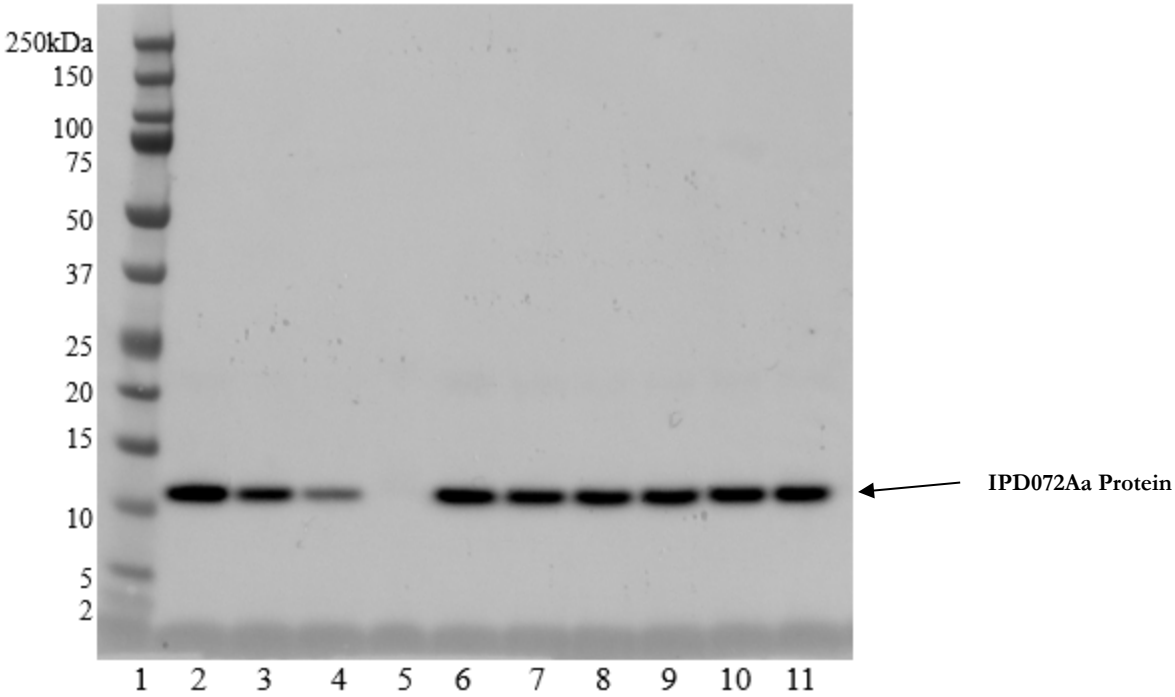
Figure 22. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3, and 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	Bioassay Control Diet (Treatment 1; Day 0)
6	Bioassay Control Diet (Treatment 1; Day 0)
7	Heat-Treated Control Diet (Treatment 5; Day 0)
8	Heat-Treated Control Diet (Treatment 5; Day 0)
9	Bioassay Control Diet (Treatment 1; Day 11)
10	Bioassay Control Diet (Treatment 1; Day 11)
11	Heat-Treated Control Diet (Treatment 5; Day 11)
12	Heat-Treated Control Diet (Treatment 5; Day 11)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. The faint bands that are barely visible in the bioassay control diet lanes are attributed to non-specific binding to diet matrix and/or artifacts of the gel loading process and are not considered indicative of contamination of the bioassay control diet and therefore not expected to have impact on study results.

Figure 23. Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 0)
7	Treatment 3 IPD072Aa Protein Test Dosing Solution (Day 0)
8	Treatment 2 IPD072Aa Protein Test Dosing Solution (Day 0)
9	Treatment 4 IPD072Aa Protein Test Dosing Solution (after final use)
10	Treatment 3 IPD072Aa Protein Test Dosing Solution (after final use)
11	Treatment 2 IPD072Aa Protein Test Dosing Solution (after final use)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective test dosing solution.

Figure 24. Assessment of the Stability of the IPD072Aa Protein in the MWM Test Dosing Solutions

F4.a. Mealworm IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the MWM bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4; 100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet wet weight, respectively. Homogeneity assessment of each of the three test diets on one day of diet preparation each is considered representative of all days of diet preparation. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively) and the stability of the IPD072Aa protein dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed.

Preparation of Solutions

Bulk dosing solutions were prepared on Day 0 of the *MWM* bioassay and maintained chilled (in a refrigerator set at 4 °C or on wet ice) until use. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.133 mg/ml, 0.667 mg/ml, and 1.33 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution for the *MWM* bioassay, samples were collected as shown in Table 132.

Table 132. MWM IPD072Aa Protein Bioassay Diet Samples

Treatment	Diet Preparation Day(s)	Number of Samples	Analysis
2	0	5 beginning	Homogeneity of IPD072Aa protein
		5 middle	
		5 end	
3	4	5 beginning	
		5 middle	
		5 end	
4	11	5 beginning	
		5 middle	
		5 end	
2	0	NA (homogeneity samples were used)	Visual confirmation of IPD072Aa protein dose
3	4		
4	11		
1	0, 11	1 from each treatment each day	Verify presence or absence of immunodetectable IPD072Aa protein
5			

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1-5 were weighed to approximately 40 mg on wet ice in preparation for SDS-PAGE. Sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). In addition, undiluted sub-samples of the test substance and sub-samples of the test dosing solutions were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance or test dosing solution, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 5 minutes. The following samples were then loaded into 4-12% Bis-Tris gels as shown in table

Table 133. MWM IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment	Day(s)	Number of Samples
Homogeneity	2	0	3 beginning
			3 middle
			3 end
	3	4	3 beginning
			3 middle
			3 end
	4	11	3 beginning
			3 middle
			3 end
Dose confirmation of IPD072Aa protein	2	0	2
	3	4	2
	4	11	2
Presence/absence of IPD072Aa protein	1	0, 11	1 per timepoint[a]
	5	0, 11	1 per timepoint[a]
Stability of IPD072Aa protein in test dosing solutions	Used to prepare Treatment 2	Day 0, after final use	1 per timepoint
	Used to prepare Treatment 3		1 per timepoint
	Used to prepare Treatment 4		1 per timepoint

[a] Samples were loaded in duplicate.

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before and after the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred

International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST four times for at least 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F4.b. Mealworm IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the Tenebrio molitor (MWM) bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera virgifera (WCR), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on Day 0 of the MWM bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing 10% Treatment 1 from the MWM bioassay by wet weight of WCR diet)
- Treatment B: Test Diet (containing 10% Treatment 4 from the MWM bioassay and targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Samples were collected from Treatments 1 and 4 on Day 0 and Day 4 diet preparation for the MWM bioassay and were used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the MWM bioassay, resulting in a 10% incorporation of the MWM diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 4 from the MWM bioassay, resulting in a 10% incorporation of the MWM diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented inTable 131. Weight data were summarized as means, standard deviations, and ranges and are presented in Table 131.

F5. Evaluation of the Survival and Weight of Superworm Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the SWM bioassay consisted of Stonefly Heliothis diet.

The carrier for the sensitive insect WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in Appendix A.

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was *Zophobas morio* (superworm; Coleoptera: Tenebrionidae SWM). SWM was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. SWM eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was WCR. The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

SWM larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 12,500 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a 12-well bioassay plate and contained 2 replicates from each treatment. Each treatment was fed to a target of 30 SWM individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed every 3 to 4 days. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control die (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatment 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4 of the SWM bioassay.

Bias in the SWM bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation

Bulk dosing solutions for Treatments 1-5 were prepared and maintained as described in the Analytical Phase section below. The boric acid dosing solution for Treatment 6 was prepared by

solubilizing boric acid then diluting individual aliquots in ultrapure water to achieve a nominal concentration of 12,500 ng/mg diet wet weight. Aliquots were maintained chilled (in a refrigerator set at 4 °C or on wet ice) until use. On each day of diet preparation, each dosing solution was mixed with carrier in a 3:1 ratio (i.e., 3 ml of dosing solution to 1 g of carrier), generating Treatments 1-6.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1-5 were collected for characterization of diets as described in the Analytical Phase section below. The positive control diet (Treatment 6) was not characterized. A portion each of Treatment 1 and Treatment 4 from Day 0 and Day 3 diet preparation was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay as described in the Sensitive Insect Bioassay section below.

SWM Bioassay

A bioassay was conducted to determine the response of SWM to IPD072Aa protein exposure via oral ingestion. SWM eggs were incubated in an environmental chamber until the eggs hatched. SWM neonates were used in the bioassay within 36 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One SWM neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 14 days. Every 3-4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living SWM larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparisons were made between SWM fed diet containing IPD072Aa (Treatment 2, 3, 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

SWM fed an artificial insect diet containing IPD072Aa protein (m_T) (Treatments 2, 3, and 4) was greater than the mortality rate of those fed the bioassay control diet (m_C) (Treatment 1). The corresponding hypothesis tests were

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight was treated as the response and analyzed using the model:

$$y_{ij} = W_i + \varepsilon_{ij}$$

where W_i denotes the mean of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} sample of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$ were independently and identically distributed. This assumption was confirmed by inspection of the residuals from the fitted model.

To test if the weight of *SWM* fed an artificial insect diet containing IPD072Aa protein (w_T) (Treatments 2, 3, and 4) was less than the weight of those fed the bioassay control diet (w_C) (Treatment 1), the corresponding hypothesis tests were

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis, and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Demonstration of IPD072Aa Protein Activity in a *SWM* Test Diet

A sensitive insect bioassay was performed using *WCR* larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 4 in the *SWM* bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section below

Results and Discussion

The SWM bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 134.

The mortality of SWM fed the test diet containing 100 ng/mg IPD072Aa protein (Treatment 2; 1 dead; 3.33% mortality; P-value = 0.7542), 500 ng/mg IPD072Aa protein (Treatment 3; 5 dead; 17.2% mortality; P-value = 0.0896), or 1000 ng/mg IPD072Aa protein (Treatment 4; 6 dead; 20.0% mortality; P-value = 0.0514), was not significantly greater than those fed the bioassay control diet (Treatment 1; 1 dead; 3.33% mortality), as shown in Table 134.

The mean weight of SWM fed Treatment 2 (0.886 mg; P-value = 0.0025), Treatment 3 (0.921 mg; P-value = 0.0109), or Treatment 4 (0.729 mg; P-value < 0.0001) was significantly less than those fed Treatment 1 (1.11 mg), as shown in Table 135.

Western blot analysis visually confirmed the homogeneity (Figure 25, Figure 26, and Figure 27) and dose (Figure 28) of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2 (Figure 29). Homogeneity assessment of each of the three test diets on one day of diet preparation each is considered representative of all days of diet preparation. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 (Figure 28) and stability of the IPD072Aa protein test dosing solutions used to prepare Treatment 2, 3, and 4 (Figure 30) were also assessed. The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 28); however, the band was less intense than that of the test diets (Treatments 2, 3, and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 28).

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 134, and Table 135. The WCR bioassay met the acceptability criterion (section F5.b. SWM IPD072Aa Protein Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in Treatment 4 of the SWM bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in section F5.b. SWM IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated exposure to a concentration of 100 ng, 500 ng, or 1000 ng IPD072Aa protein per mg diet (Treatments 2, 3, and 4, respectively) had no adverse effect on survival of SWM. No statistically significant differences were observed in mortality between SWM fed Treatments 2, 3, and 4 (3.33%, 17.2%, and 20.0%, respectively) and those fed the bioassay control diet (Treatment 1; 3.33%). A statistically significant difference was observed in mean weight between SWM fed Treatments 2, 3, and 4 (0.886 mg, 0.921 mg, and 0.729 mg, respectively) and those fed Treatment 1 (1.11 mg).

Table 134. Summary Analysis of SWM IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	30	1	3.33	0.7542
3	Test Diet	500	29[a]	5	17.2	0.0896
4	Test Diet	1000	30	6	20.0	0.0514
5	Heat-treated Control Diet	1000	30	2	6.67	--
6	Positive Control Diet	0	29[a]	29	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight. Treatment 6 contained a targeted concentration of 12,500 ng boric acid per mg diet wet weight.

[a] Organisms counted as missing during the bioassay, or wells containing more than one organism, were not included in the total number of observations for a given treatment.

Table 135. Summary Analysis of SWM IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	29	1.11 (1.00 - 1.22)	0.7 - 2.1	--
2	Test Diet	100	28	0.886 (0.773 - 0.998)	0.4 - 1.6	0.0025[a]
3	Test Diet	500	24	0.921 (0.799 - 1.04)	0.4 - 1.4	0.0109[a]
4	Test Diet	1000	24	0.729 (0.608 - 0.851)	0.4 - 1.2	<0.0001[a]
5	Heat-treated Control Diet	1000	28	1.20 ± 0.340[b]	0.5 - 2.2	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 12,500 ng boric acid per mg diet wet weight. Not applicable (NA); there were no surviving *Zophobas morio* in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

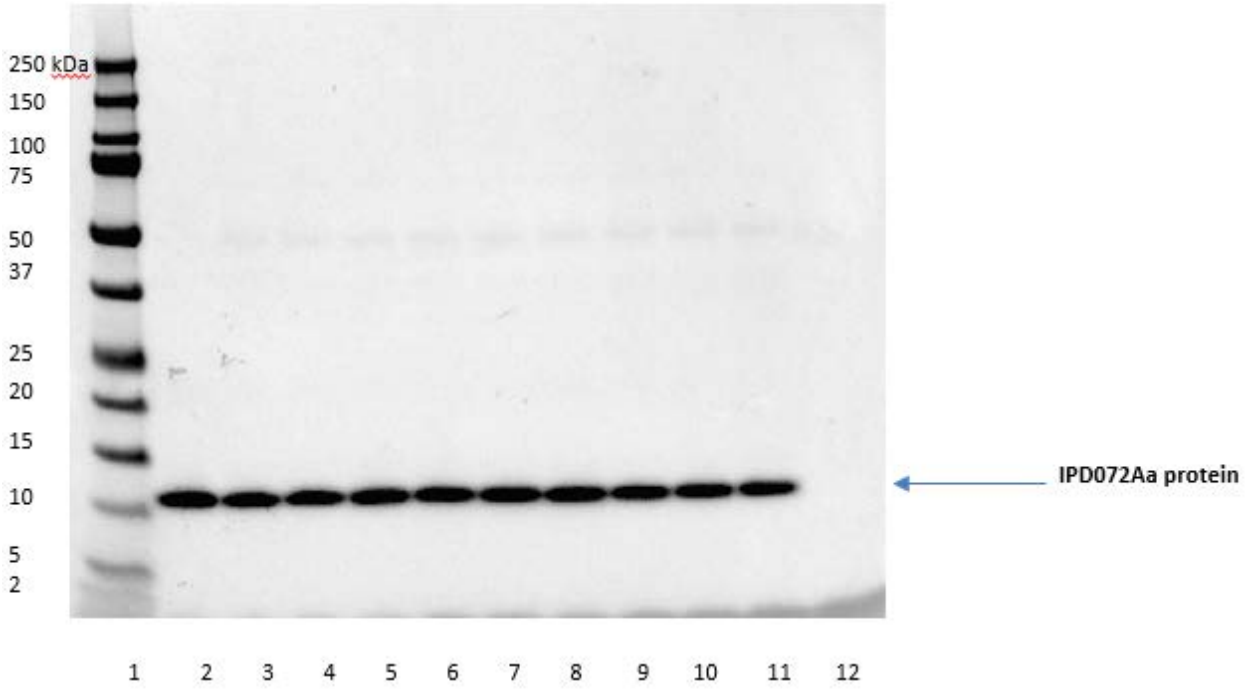
[b] Standard deviation is provided for mean values not subjected to Fisher's exact test.

Table 136. Summary of SWM IPd072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	29[a]	13.8	25	0.368 ± 0.141	0.1 - 0.6
B	Test Diet	100	30	90.0	3	0.100 ± 0.00	0.1 - 0.1

Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared from the same diet preparations used in Treatments 1 and 4, respectively, of the SWM bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

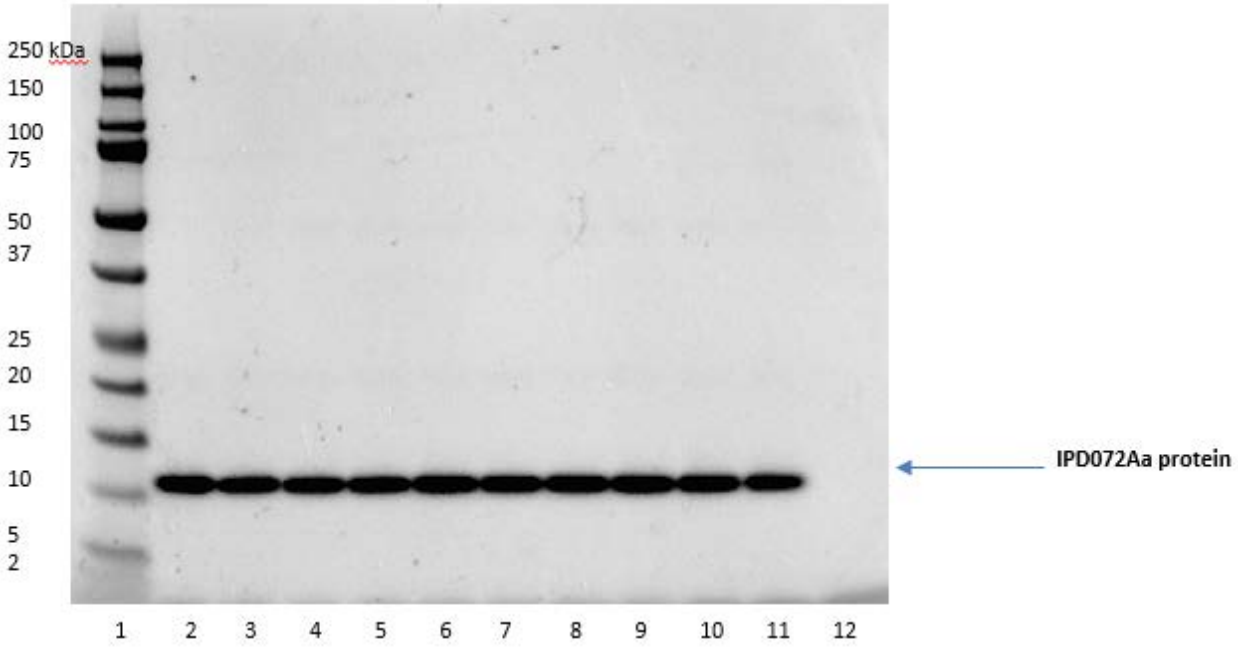
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet distribution process at which the samples were collected.

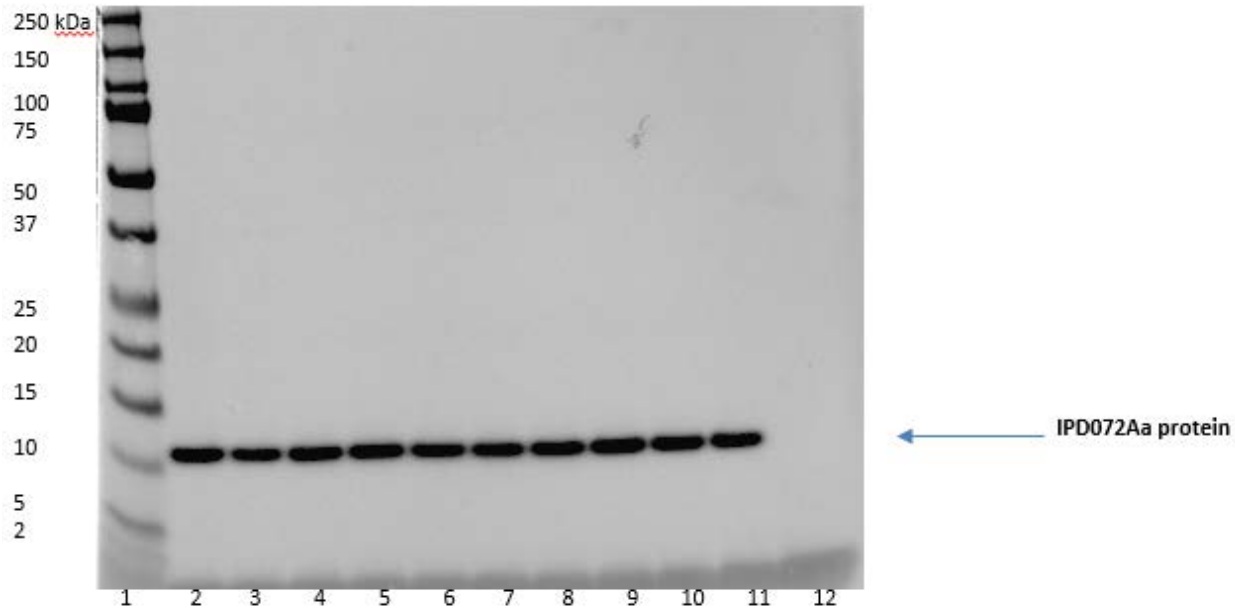
Figure 25. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2, Day 0



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; End)
10	IPD072Aa Protein Test Diet (Treatment 3; End)
11	IPD072Aa Protein Test Diet (Treatment 3; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 3. Beginning, middle, and end refer to the stages in the diet distribution process at which the samples were collected.

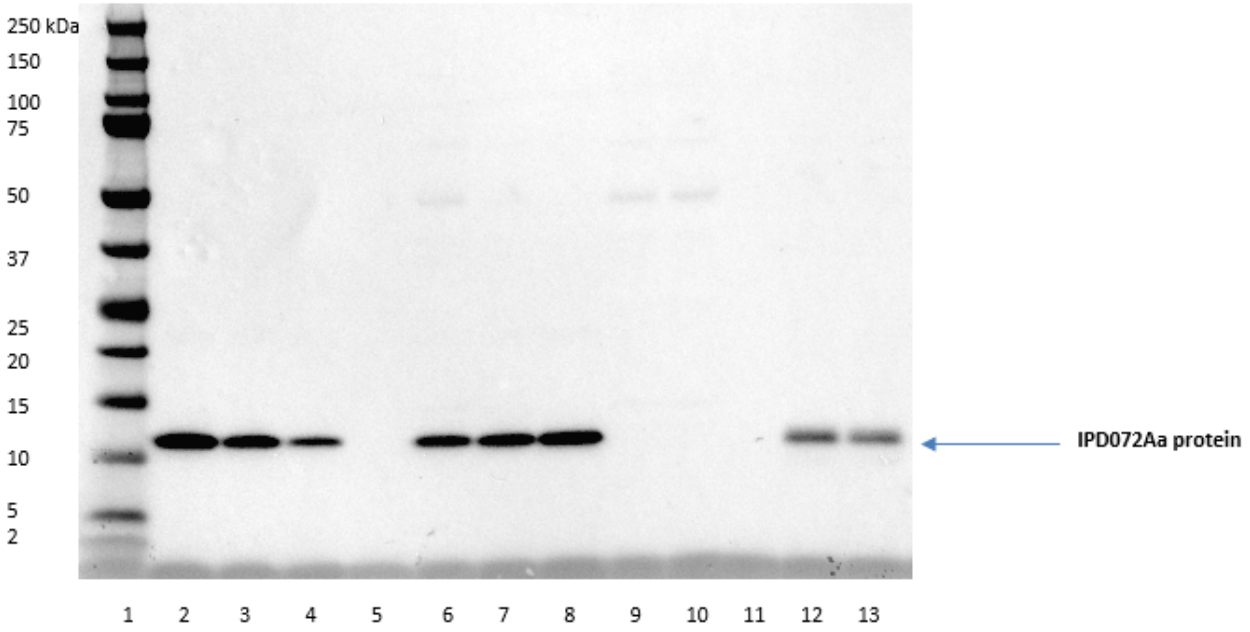
Figure 26. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3, Day 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stages in the diet distribution process at which the samples were collected.

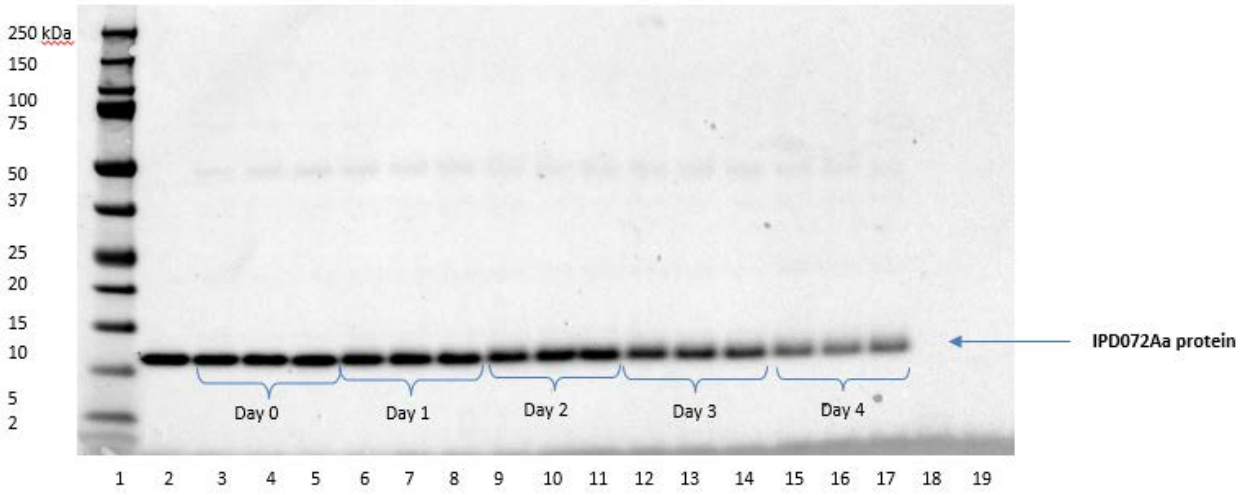
Figure 27. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4, Day 11



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
7	IPD072Aa Protein Test Diet (Treatment 3; Day 3)
8	IPD072Aa Protein Test Diet (Treatment 4; Day 11)
9	Bioassay Control Diet (Treatment 1; Day 0)
10	Bioassay Control Diet (Treatment 1; Day 11)
11	1X LDS Sample Buffer Blank
12	Heat-Treated Control Diet (Treatment 5; Day 0)
13	Heat-Treated Control Diet (Treatment 5; Day 11)

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatments 2, 3, and 4 were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

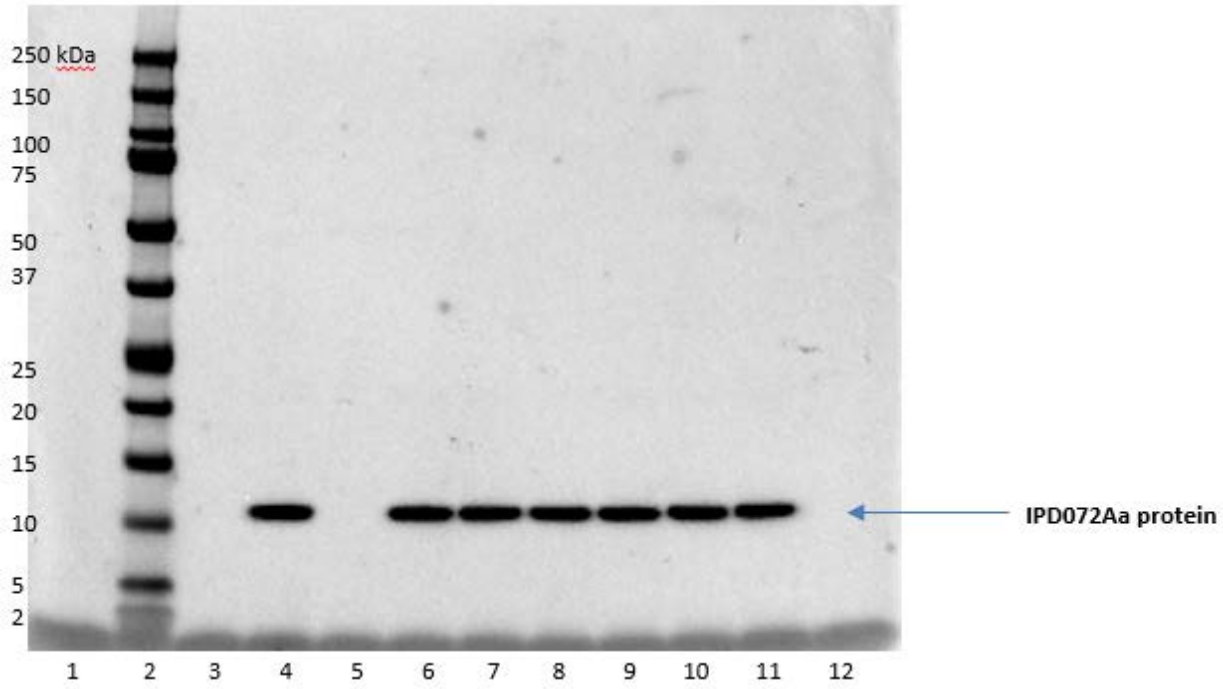
Figure 28. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3, and 4, and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
2	Test Substance (10 ng)	12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	18	1X LDS Sample Buffer Blank
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	19	1X LDS Sample Buffer Blank
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)		

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Days 0-4 refer to the number of days for which samples were stored under bioassay conditions prior to analysis. On Days 3 and 4 of stability analysis, the IPD072Aa protein bands appeared less intense and more diffuse than on Days 0-2; however, organisms were refed on Day 3 or Day 4.

Figure 29. Stability Assessment of the IPD072Aa Protein in Treatment 2 under Bioassay Conditions



Lane	Sample Identification
1	1X LDS Sample Buffer Blank
2	Pre-stained Protein Molecular Weight Markers
3	1X LDS Sample Buffer Blank
4	Test Substance (10 ng)
5	1X LDS Sample Buffer Blank
6	Treatment 2 IPD072Aa Protein Test Dosing Solution (Day 0)
7	Treatment 2 IPD072Aa Protein Test Dosing Solution (after final use)
8	Treatment 3 IPD072Aa Protein Test Dosing Solution (Day 0)
9	Treatment 3 IPD072Aa Protein Test Dosing Solution (after final use)
10	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 0)
11	Treatment 4 IPD072Aa Protein Test Dosing Solution (after final use)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective test dosing solution.

Figure 30. Stability of the IPD072Aa Protein in the Test Dosing Solutions

F5.a. Superworm IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the SWM bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diets (Treatments 2, 3, and 4; 100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet wet weight, respectively). Homogeneity assessment of one test diet on one day of diet preparation is considered representative of all days of diet preparation for all test diets. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, stability under bioassay conditions of the IPD072Aa protein in Treatment 2, and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed.

Preparation of Solutions

Bulk dosing solutions were prepared on Day 0 of the SWM bioassay and maintained chilled (in a 4 °C refrigerator unit or on wet ice) until use. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.133 mg/ml, 0.667 mg/ml, and 1.33 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution for the SWM bioassay, samples were collected as shown in **Table 137**

Table 137. SWM IPD072Aa Protein Bioassay Diet Samples

Treatment	Diet Preparation Day(s)	Number of Samples	Analysis
2	0	5 beginning	Homogeneity of IPD072Aa protein
		5 middle	
		5 end	
3	3	5 beginning	
		5 middle	
		5 end	
4	11	5 beginning	
		5 middle	
		5 end	
2	0	NA (homogeneity sample was used)	Visual confirmation of IPD072Aa protein dose
3	3	NA (homogeneity sample was used)	
4	11	NA (homogeneity sample was used)	
1	0, 11	1 from each treatment each day	Verify presence or absence of immunodetectable IPD072Aa protein
5			
1	3	5 (for Day 0 assessment)	Oven dry weight equivalence
		20 (distributed to bioassay plates)	
2	0	NA (homogeneity samples were used for Day 0 assessment)[a]	Stability under bioassay conditions
	3	20 (distributed to bioassay plates)	

[a] A Day 0 homogeneity sample for Treatment 2 is representative of Day 0 stability assessment for Treatment 2.

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, the samples of Treatment 1 and Treatment 2 (20 each) that had been distributed to bioassay plates were placed under bioassay conditions using the same methods and conditions used in the SWM bioassay, except they were not infested with larvae. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Days 1-4), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A

combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1-5 were weighed to approximately 40 mg on wet ice in preparation for SDS-PAGE. For dose confirmation and assessment of homogeneity in Treatments 2, 3, and 4, Day 0 stability under bioassay conditions, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance and sub-samples of the test dosing solutions (Day 0 and following final use) were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance or test dosing solution, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 5 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown in Table 138.

Table 138. SWM IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment	Day(s)	Number of Samples
Dose confirmation of IPD072Aa protein	2	0	1
	3	3	1
	4	11	1
Homogeneity	2	0	3 beginning
			3 middle
			3 end
	3	3	3 beginning
			3 middle
			3 end
	4	11	3 beginning
			3 middle
			3 end
Stability under bioassay conditions	2	0-4	3 per timepoint
Stability of IPD072Aa protein in test dosing solutions	Used to prepare Treatment 2	Day 0, after final use	1 per timepoint
	Used to prepare Treatment 3		1 per timepoint
	Used to prepare Treatment 4		1 per timepoint
Presence/absence of IPD072Aa protein	1	0, 11	1 per timepoint
	5		1 per timepoint

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 52-80 minutes at ambient temperature. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v

non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST three times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST three times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F5.b. SWM IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the SWM bioassay was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on Day 0 of the SWM bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing 10% Treatment 1 from the SWM bioassay by wet weight of WCR diet)
- Treatment B: Test Diet (containing 10% Treatment 4 from the SWM bioassay and targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Samples were collected from Treatments 1 and 4 on Day 0 and Day 3 diet preparation for the SWM bioassay and were used to prepare Treatments A and B (Day 0 and Day 3 diet preparation) for the WCR bioassay as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the SWM bioassay, resulting in a 10% incorporation of the SWM diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 4 from the SWM bioassay, resulting in a 10% incorporation of the SWM diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 3, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 136. Weight data were summarized as means, standard deviations, and ranges and are presented inTable 136.

F6. Evaluation of the Survival and Weight of Red Flour Beetle Fed an Artificial Diet Containing Purified IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the *RFB* bioassay consisted of red flour beetle diet prepared by Pioneer Hi-Bred International, Inc

The carrier for the WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution consisted of ultrapure water.

The test dosing solution consisted of the test substance diluted in ultrapure water to achieve the concentration in the test diet (Treatment 2).

The heat-treated control dosing solution consisted of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes as detailed in section F6.a. Red Flour Beetle IPD072Aa Bioassay Analytical Phase.

The positive control dosing solution consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was *Tribolium castaneum* (red flour beetle; Coleoptera: Tenebrionidae; RFB). RFB was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. RFB eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was Diabrotica virgifera virgifera (western corn rootworm; Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

RFB larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 4: Positive Control Diet (targeting 10,000 ng boric acid per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 5 blocks. Each block consisted of a tray containing 1-oz plastic cups and contained 6 replicates from each treatment. Each treatment was fed to a target of 30 RFB individuals. The bioassay was conducted in an environmental chamber set at 30 °C, 70% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between RFB provided Treatments 1 and 2.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 4) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose, homogeneity, freezer stability, and stability under bioassay conditions of the IPD072Aa protein in the test diet. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein used in the RFB test diet (Treatment 2).

Bias in the RFB bioassay and sensitive insect bioassay portions of this study was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1, 2, and 3 were prepared as described in the Analytical Phase section below. The boric acid dosing solution for Treatment 4 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 10,000 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 2:1 ratio (i.e., 2 ml dosing solution to 1 g carrier) to generate Treatments 1-4. After mixing, diets were transferred to 50-ml tubes, lyophilized, finely homogenized, and then pooled together by treatment. The bulk diets were aliquoted into individual storage tubes and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1, 2, and 3 were collected for use in characterization of diets as described in the Analytical Phase section below. The positive control diet (Treatment 4) was not characterized. A portion of Treatment 1 and 2 was collected for use in diets in the sensitive insect bioassay as described in the Sensitive Insect Bioassay section below.

RFB Bioassay

A bioassay was conducted to determine the response of RFB to IPD072Aa protein exposure via oral ingestion. RFB eggs were incubated in an environmental chamber until the eggs hatched. RFB neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 8 mg of diet were distributed into individual plastic cups. One RFB neonate was placed in each cup containing diet, and then the cup was sealed with a lid. The bioassay was conducted in an environmental chamber set at 30 °C, 70% relative humidity, and a 24-hour dark cycle for a total of 7 days. On Day 3, new bioassay cups were prepared as described for Day 0. Living RFB larvae were transferred to the new cups, missing or dead larvae were recorded, and the freshly prepared cups were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only cups that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a cup, or cups containing more than one organism, were excluded from reporting.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparisons were made between RFB fed diet containing IPD072Aa protein (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to compare if the mortality rate of RFB fed the artificial insect diet containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

Significance was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption necessary for a two-sample t-test was not satisfied by data distributions of Treatment 1 and 2; therefore, a non-parametric Wilcoxon two-sample test was conducted to test if exposure to artificial insect diet containing IPD072Aa protein caused growth inhibition. That is, to test if the weight of RFB fed the artificial insect diet containing IPD072Aa protein (w_T) was less than the weight of those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by a non-parametric Siegel-Tukey test; therefore, the Wilcoxon test was valid. Significance was established if the P-value was < 0.05. SAS PROC NPAR1WAY was used to conduct both tests.

Demonstration of IPD072Aa Protein Activity in the *RFB* Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 2 in the RFB bioassay. Details regarding the sensitive insect bioassay are provided section F6.b. Red Flour Beetle IPD072Aa Protein Sensitive Insect Bioassay.

Results and Discussion

The RFB bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 139.

The mortality of RFB fed the diet containing IPD072Aa protein (Treatment 2; 1 dead; 3.57% mortality) was not significantly greater than those fed the bioassay control diet (Treatment 1; 1 dead; 3.57%; Fisher's exact test P-value = 0.7545), as shown in Table 139.

The mean weight of RFB fed the diet containing IPD072Aa protein (Treatment 2; 0.304 mg) was not significantly less than those fed the bioassay control diet (Treatment 1; 0.370 mg; Wilcoxon test P-value = 0.0881), as shown in Table 140.

Western blot analysis visually confirmed the dose, homogeneity, freezer stability, and stability under bioassay conditions of the IPD072Aa protein in the test diet (Treatment 2; Figure 31, Figure 32, and Figure 33). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 3, Figure 31), however, the band was less intense than that of the test diet (Treatment 2). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 31).

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 3. The WCR bioassay met the acceptability criterion (Sensitive Insect Bioassay section). The biological activity of the IPD072Aa protein in the test diet used in the RFB bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described section F6.b. Red Flour Beetle IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on survival and weight of RFB.

Table 139. Summary Analysis of RFB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	28[a]	1	3.57	0.7545
2	Test Diet	28[a]	1	3.57	
3	Heat-treated Control Diet	29[a]	0	0	--
4	Positive Control Diet	29[a]	29	100	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet dry weight.
[a]Organisms counted as missing during the bioassay, or cups containing more than one organism, were not included in the total number of observations for a given treatment.

Table 140. Summary Analysis of RFB IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean ± Standard Deviation (mg)	Range (mg)	Wilcoxon P-Value
1	Bioassay Control Diet	27	0.370 ± 0.179	0.1 - 0.9	0.0881
2	Test Diet	27	0.304 ± 0.143	0.1 - 0.7	
3	Heat-treated Control Diet	29	0.376 ± 0.143	0.1 - 0.6	--
4	Positive Control Diet	0	NA	NA	--

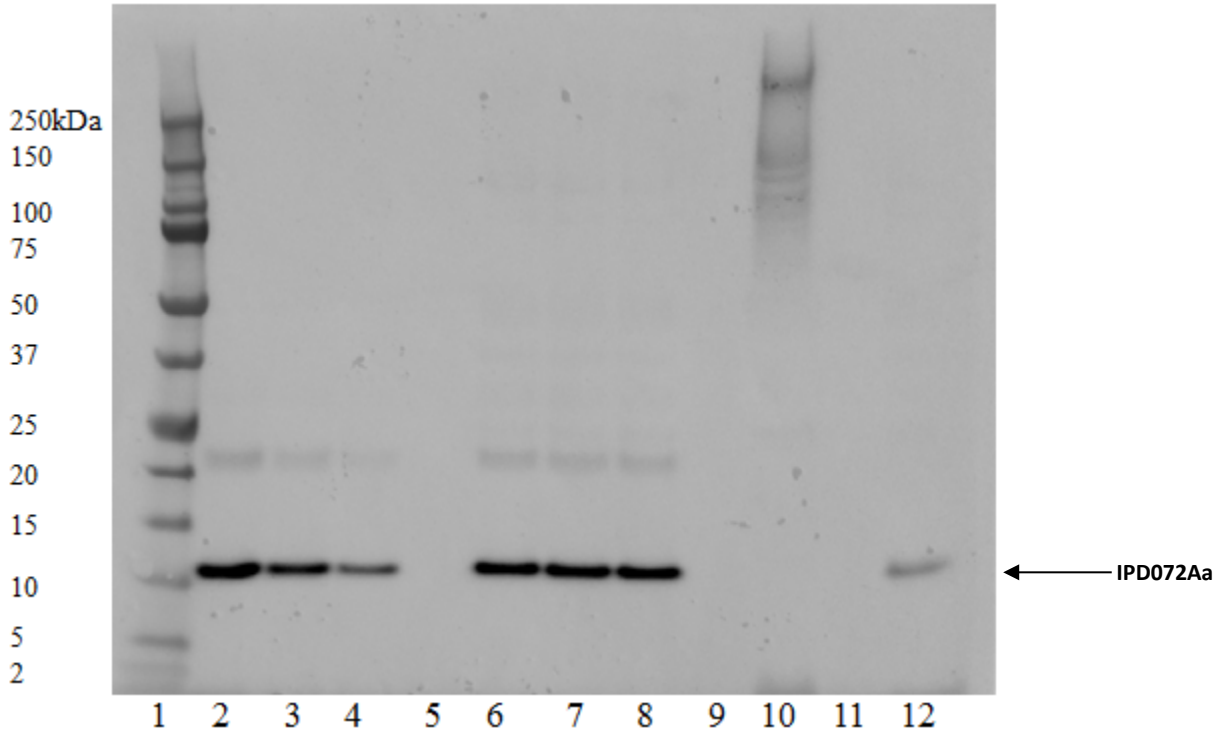
Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet dry weight.

Table 141. Summary of RFB IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0 ng IPD072Aa/mg	29[a]	10.3	26	0.523 ± 0.111	0.300 - 0.700
B	Test Diet	57 ng IPD072Aa/mg (wet weight)	29[a]	75.9	7	0.114 ± 0.0690	0.00 - 0.200

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera)* bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 2, respectively, of the *Tribolium castaneum* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the corn rootworm artificial diet.

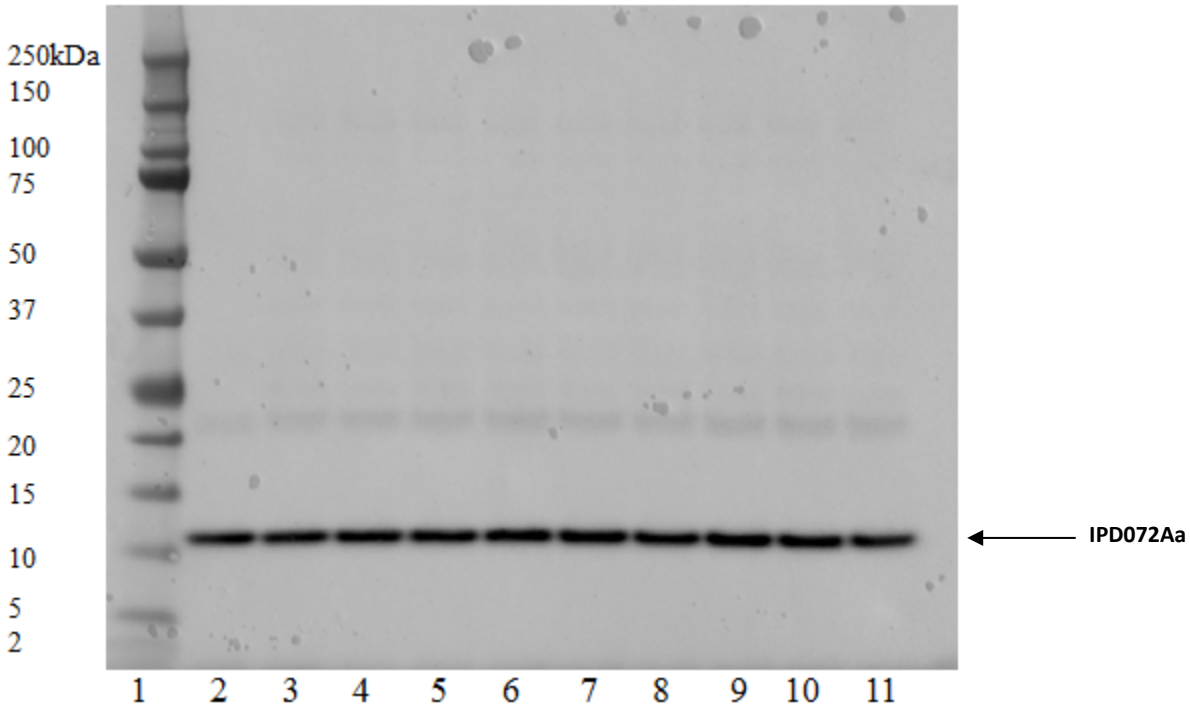
[a]Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	7	IPD072Aa Protein Test Diet (Treatment 2; Freezer Stability Sample Rep1; 10 ng)
2	Test Substance (20 ng)	8	IPD072Aa Protein Test Diet (Treatment 2; Freezer Stability Sample Rep2; 10 ng)
3	Test Substance (10 ng)	9	1X LDS Sample Buffer Blank
4	Test Substance (5 ng)	10	Bioassay Control Diet (Treatment 1; undiluted)
5	1X LDS Sample Buffer Blank	11	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; 10 ng)	12	Heat-Treated Control Diet (Treatment 3; 10 ng)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test, freezer stability, and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lane 10 are attributed to non-specific binding to diet components in the more concentrated bioassay control diet analytical sample (~30x relative to Lanes 6, 7, 8, and 12).

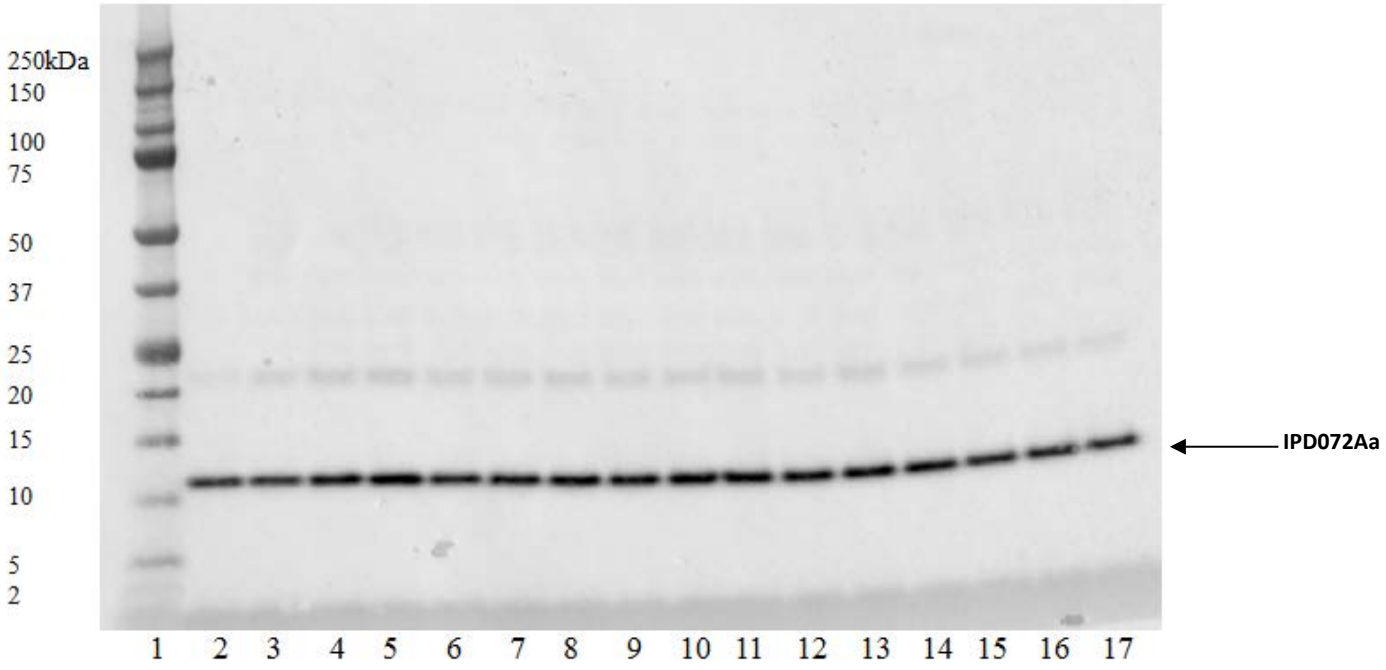
Figure 31. Dose Confirmation and Freezer Stability of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in the Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3)



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
2	Test Substance (10 ng)	8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)	9	IPD072Aa Protein Test Diet (Treatment 2; End)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)	10	IPD072Aa Protein Test Diet (Treatment 2; End)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)	11	IPD072Aa Protein Test Diet (Treatment 2; End)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)		

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). Beginning, middle, and end refer to the stage in the diet aliquoting process at which samples were collected.

Figure 32. Homogeneity Assessment of the IPD072Aa Protein in the Test Diet (Treatment 2)



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
2	Test Substance (10 ng)	11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)		

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). Days 0-4 refers to the number of days samples were stored under bioassay conditions prior to analysis.

Figure 33. Stability Assessment of the IPD072Aa Protein in the Test Diet (Treatment 2) under Bioassay Conditions

F6.a. Red Flour Beetle IPD072Aa Bioassay Analytical Phase

The following dosing solutions were prepared for the RFB bioassay:

- Bioassay control dosing solution consisting of chilled ultrapure water (defined as American Society for Testing and Materials ASTM Type 1 water)

- Test dosing solution consisting of IPD072Aa protein test substance diluted in ultrapure water

- Heat-treated IPD072Aa protein control dosing solution consisting of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose (1000 ng IPD072Aa protein per mg diet dry weight), homogeneity, freezer stability, and stability under bioassay conditions of the IPD072Aa protein in the test diet (Treatment 2 in the *RFB* bioassay). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 3, respectively, in the *RFB* bioassay) was also assessed.

Preparation of Solutions

The test dosing solution was prepared on the day of diet preparation. To generate the test dosing solution, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to an IPD072Aa protein concentration of 0.5 mg/ml. To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the *RFB* bioassay, samples of Treatments 1, 2, and 3 were collected as follows:

On Day 0 of the diet distribution process, 15 samples of Treatment 2 were collected, five each from the beginning, middle, and end of the diet aliquoting process, to assess homogeneity of the IPD072Aa protein in the test diet. Some of the samples collected for homogeneity were

also used for visual confirmation of the dose of IPD072Aa protein in the test diet and Day 0 stability under bioassay conditions.

On Day 0 of the diet distribution process, one bulk aliquot of Treatment 2 was collected and stored frozen (-80 °C freezer unit) to assess stability after final use in the bioassay.

On Day 0 of the diet distribution process, an aliquot each of Treatment 1 and Treatment 3 were collected to verify the presence or absence of immunodetectable IPD072Aa protein.

Five samples of Treatment 1 were collected and used as Day 0 samples for oven dry weight equivalence.

A bulk aliquot each of Treatment 1 and Treatment 2 were collected to assess stability under bioassay conditions and stored frozen (-80 °C freezer unit) until bioassay stability initiation.

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, the aliquots of Treatment 1 and Treatment 2 were removed from storage, distributed to cups (20 for each treatment), and placed under bioassay conditions using the same methods and conditions used in the *RFB* bioassay, with the exception that extra diet was dispensed to cups to ensure there was enough diet to recover for analytical purposes. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Day 1, 2, 3, and 4), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability of Treatment 2 under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and SDS-PAGE

Sub-samples of Treatments 1, 2, and 3 were weighed to approximately 10 mg each on dry ice in preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For dose confirmation, assessment of homogeneity, and freezer stability in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3, sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, an undiluted sub-sample of the test substance was prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed, diluted, as applicable, heated at 90-100 °C for 5 minutes, and then stored frozen (-80 °C freezer unit). Prior to analysis, samples were heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as follows:

For visual confirmation of the dose and freezer stability of IPD072Aa protein in the test diet and assessment of the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3, dilutions of the test substance were loaded (5, 10, and 20 ng) to a gel. Treatment 2 and Treatment 3 (10 ng each based on nominal concentration) were also loaded to the gel. Treatment 1 was loaded to the gel undiluted.

For homogeneity assessment, nine samples of Treatment 2 (three each from the beginning, middle, and end of the diet aliquoting process) and a sample of the test substance were loaded to a gel at 10 ng each.

For assessment of stability under bioassay conditions, Treatment 2 was loaded to a gel at 10 ng (based on nominal concentration of Treatment 2) for each sampling time point (Days 0-4). A sample of the test substance (10 ng) was also loaded.

Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST for at least 1 minute to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:15,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Protein aggregation such as dimer formation may be observed; an IPD072Aa protein dimer would be expected to migrate at approximately 20 kDa.

F6.b. Red Flour Beetle IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 2 used in the Tribolium castaneum (RFB) bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera virgifera (WCR), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after completion of the RFB bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 20% Treatment 1 from the RFB bioassay by dry weight of WCR diet)

Treatment B: Test Diet (containing 20% Treatment 2 from the RFB bioassay by dry weight of diet; targeting 57 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 *WCR* individuals.

Diet Preparation

Stored aliquots of Treatments 1 and 2 from the RFB bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B as follows:

For Treatment A, corn rootworm artificial diet was mixed with Treatment 1 from the RFB bioassay, resulting in a 20% incorporation of the RFB diet by dry weight of the WCR diet.

For Treatment B, corn rootworm artificial diet was mixed with Treatment 2 from the RFB bioassay, resulting in a 20% incorporation of the RFB diet by dry weight of the WCR diet.

Then, for each respective treatment, ultrapure (American Society for Testing and Materials (ASTM) Type 1) water was mixed with the dry ingredients at a 2.5:1 ratio (i.e., 2.5 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from reporting.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 141. Weight data is summarized as means, standard deviations, and ranges in Table 141.

F7. Evaluation of the Survival and Weight of Mexican Bean Beetle Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the MBB bioassay consisted primarily of Stonefly Heliothis diet.

The carrier for the sensitive insect WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section F7.a. Mexican Bean Beetle IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was *Epilachna varivestis* (Mexican bean beetle; Coleoptera: Coccinellidae; MBB). MBB was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. MBB eggs were obtained from New Jersey Department of Agriculture (Trenton, NJ, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was WCR. The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

MBB larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 6: Positive Control Diet (targeting 1250 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 15 blocks. Each block consisted of a 12-well bioassay plate and contained two replicates from each treatment. Each treatment was fed to a target of 30 MBB individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.

The mortality of the positive control diet (Treatment 6) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2 and 4 and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the MBB bioassay.

Bias in the MBB bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Bulk dosing solutions for Treatments 1-5 were prepared and maintained as described in the Analytical Phase section below. The boric acid dosing solution for Treatment 6 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 1250 ng/mg diet wet weight. On each day of diet preparation, each dosing solution was mixed with carrier in a 3:1 ratio (i.e., 3 ml of dosing solution to 1 g of carrier), generating Treatments 1-6.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1, 2, 4, and 5 were collected for characterization of diets as described in the Analytical Phase section below. Treatment 3 and the positive control diet (Treatment 6) were not characterized. A portion each of Treatments 1 and 4 from each day of diet preparation was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay as described in the section F7.b. Mexican Bean Beetle IPD072Aa Protein Sensitive Insect Bioassay.

MBB Bioassay

A bioassay was conducted to determine the response of MBB to IPD072Aa protein exposure via oral ingestion. MBB eggs were incubated in an environmental chamber until the eggs hatched. MBB neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One MBB neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 7 days. On Day 3, new bioassay plates were prepared with fresh diet as described for Day 0, living MBB larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparisons were made between MBB fed diet containing IPD072Aa protein (Treatments 2, 3, and 4) and the bioassay control diet (Treatment 1) for mortality. Due to high mortality in Treatments 3 and 4, statistical comparison was made only between Treatment 2 and Treatment 1 for weight.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

MBB fed an artificial insect diet containing IPD072Aa protein (m_T) (Treatments 2, 3, and 4) was greater than the mortality rate of those fed the bioassay control diet (m_C) (Treatment 1). The corresponding hypothesis test was

$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0$.

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight was treated as the response and analyzed using the model:

$$y_{ij} = W_i + \varepsilon_{ij}$$

where W_i denotes the mean of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} sample of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$ were independently and identically distributed. This assumption was confirmed by inspection of the residuals from the fitted model.

To test if the weight of MBB fed an artificial insect diet containing IPD072Aa protein (w_T) (Treatment 2) was less than the weight of those fed the bioassay control diet (w_C) (Treatment 1), the corresponding hypothesis tests were

$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0$.

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis, and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Demonstration of IPD072Aa Protein Activity in the MBB Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of IPD072Aa protein in Treatment 4 in the MBB bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section below.

Results and Discussion

The MBB bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 6) group exceeded 80%, as shown in Table 142.

The mortality of MBB fed the test diet containing 100 ng/mg IPD072Aa protein (Treatment 2; 5 dead; 16.7% mortality; P-value = 0.0973) was not significantly greater than those fed the bioassay control diet (Treatment 1; 1 dead; 3.33% mortality), as shown in Table 142. The mortality of MBB fed the test diets containing 500 ng/mg and 1000 ng/mg IPD072Aa protein (Treatments 3 and 4; 28 and 30 dead and 96.6% and 100% mortality, respectively; P-values < 0.0001) was significantly greater than those fed the bioassay control diet (Treatment 1), as shown in Table 142.

The mean weight of MBB fed Treatment 2 (0.436 mg; t-test P-value < 0.0001) was significantly less than those fed Treatment 1 (1.23 mg), as shown in Table 143. Mean weight was not calculated for MBB fed Treatments 3 and 4 due to one or no surviving larvae in each treatment group.

Western blot analysis visually confirmed the homogeneity and the dose of the IPD072Aa protein in Treatments 2 and 4 (Figure 34Figure 35Figure 37) and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2 (Figure 36). Homogeneity assessment of two test diets on one day of diet preparation each is considered representative of both days of diet preparation for all test diets. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 and stability of the IPD072Aa protein test dosing solutions used

to prepare Treatments 2, 3, and 4 were also assessed (Figure 37). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 37); however, the band was less intense than that of the test diets (Treatments 2 and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 37).

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 142. The WCR bioassay did not meet the acceptability criterion; however, the test substance indicated bioactivity in the MBB bioassay and therefore, the WCR bioassay was not repeated (Sensitive Insect Bioassay section). The biological activity of the IPD072Aa protein in Treatment 4 of the MBB bioassay was further demonstrated by 100% mortality in WCR fed the test diet (Treatment B; described in section F7.b. Mexican Bean Beetle IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated exposure to a concentration of 100 ng IPD072Aa protein per mg diet (Treatment 2) had no adverse effect on survival of MBB. No statistically significant differences were observed in mortality between MBB fed Treatment 2 (16.7%) and those fed the bioassay control diet (Treatment 1; 3.33%). A statistically significant difference was observed in mean weight between MBB fed Treatment 2 (0.436 mg) and those fed Treatment 1 (1.23 mg). Statistically significant differences in mortality were observed between MBB fed diets containing 500 ng/mg or 1000 ng/mg IPD072Aa protein (Treatments 3 and 4, 96.6% and 100%, respectively) and those fed Treatment 1.

Table 142. Summary Analysis of MBB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	30	5	16.7	0.0973
3	Test Diet	500	29[a]	28	96.6	<0.0001[b]
4	Test Diet	1000	30	30	100	<0.0001[b]
5	Heat-treated Control Diet	1000	30	0	0	--
6	Positive Control Diet	0	30	30	100	--.

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet wet weight. Treatment 6 contained a targeted concentration of 1250 ng boric acid per mg diet wet weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed.

Table 143. Summary Analysis of MBB IPD072Aa Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	29	1.23 (1.14 - 1.33)	0.7 - 1.9	--
2	Test Diet	100	25	0.436 (0.333 - 0.539)	0.2 - 0.8	<0.0001[a]
3	Test Diet	500	1	0.5[b]	NA	--
4	Test Diet	1000	0	NA	NA	--
5	Heat-treated Control Diet	1000	30	1.27 ± 0.325[c]	0.6 - 2.3	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet wet weight. Treatment 6 contains a targeted concentration of 1250 ng boric acid per mg diet wet weight. Not applicable (NA); there were no surviving *Epilachna varivestis* in Treatment 4 or Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] The reported mean is the weight value of the one surviving larva.

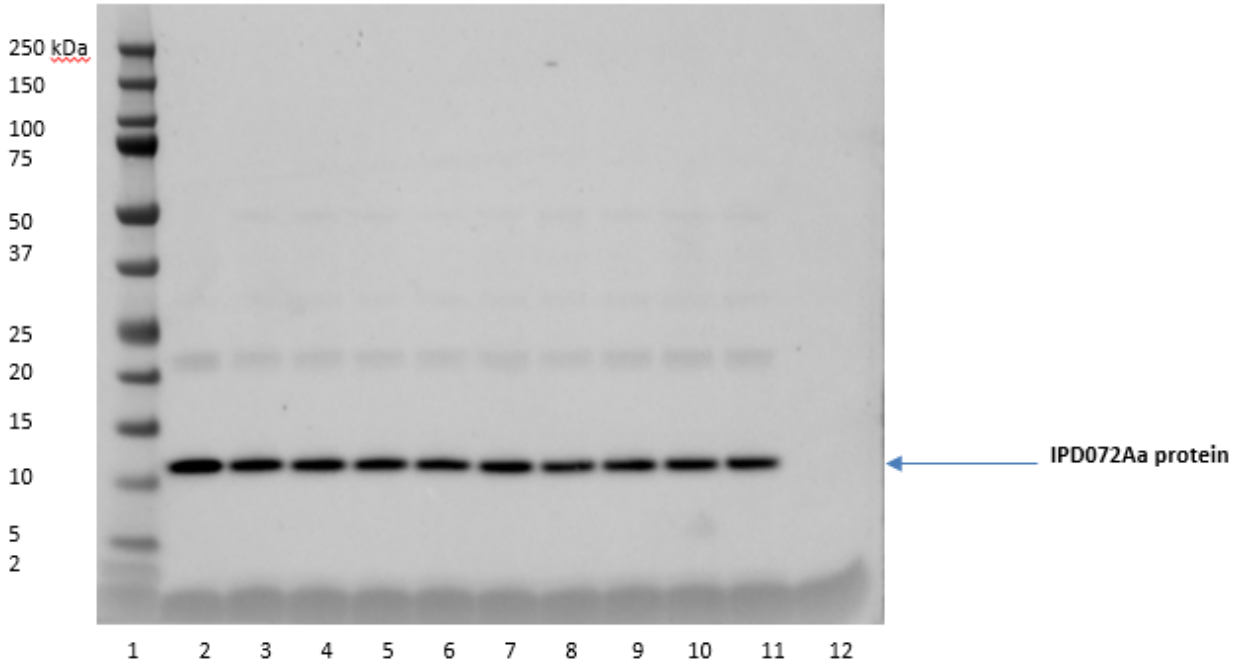
[c]Standard deviation is provided for mean values not subjected to a *t*-test.

Table 144. Summary of MBB IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0	26[a]	6	23.1
B	Test Diet	100	29[a]	29	100

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera)* bioassay were prepared from the same diet preparations used in Treatments 1 and 4, respectively, of the *Epilachna varivestis* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

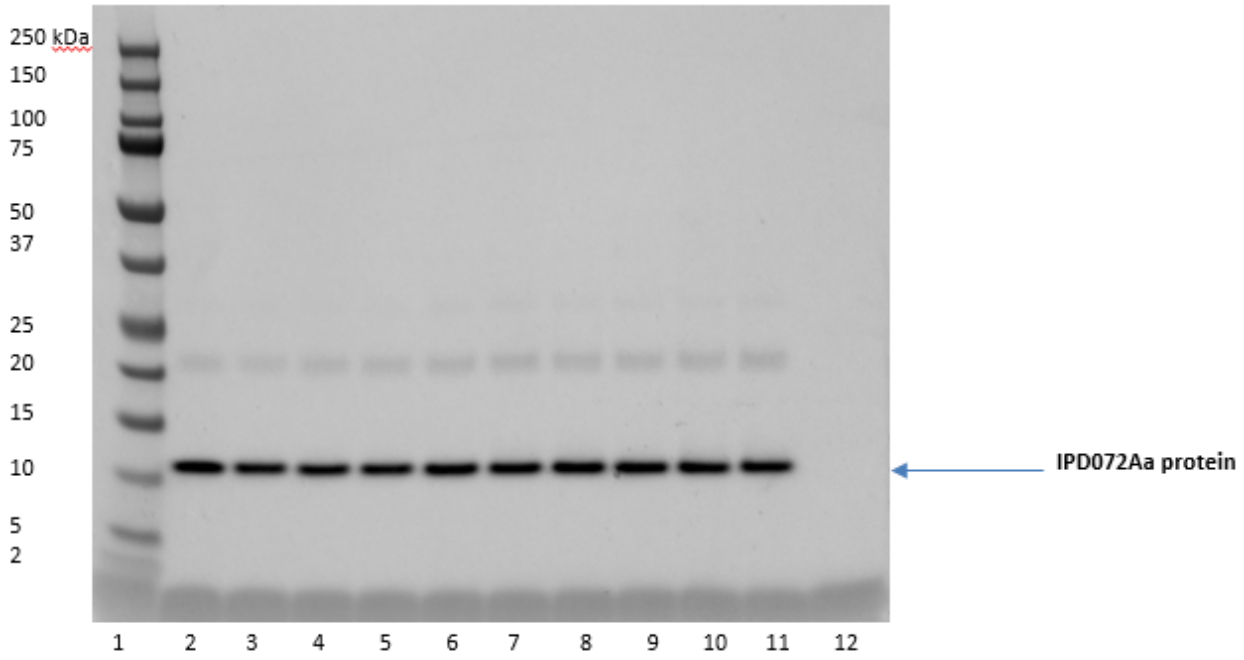
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

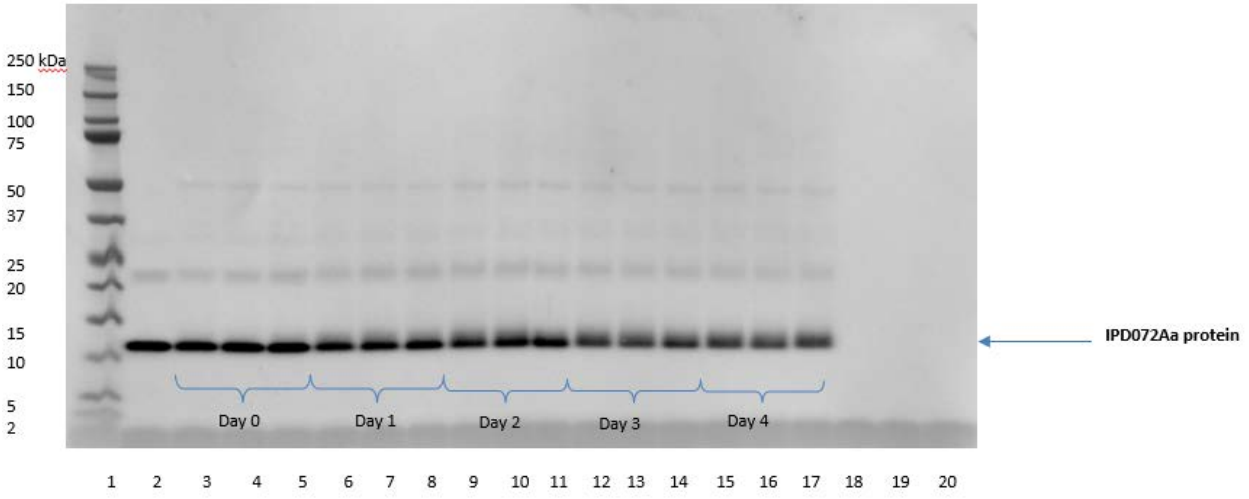
Figure 34. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2, Day 0



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

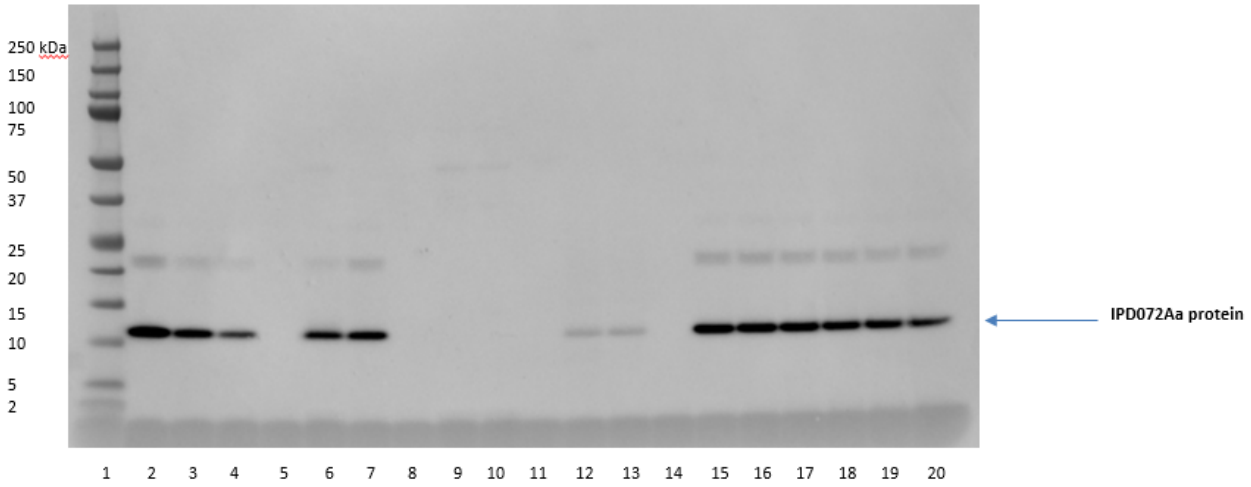
Figure 35. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4, Day 3



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker	11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
2	Test Substance (10 ng)	12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	18	1X LDS Sample Buffer Blank
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	19	1X LDS Sample Buffer Blank
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	20	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Days 0-4 refer to the number of days for which samples were stored under bioassay conditions prior to analysis. On Days 3 and 4 of stability analysis, the IPD072Aa protein bands appeared less intense and more diffuse than on Days 0-2; however, organisms were refed on Day 3.

Figure 36. Stability Assessment of the IPD072Aa Protein in Treatment 2 under Bioassay Conditions



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker	11	1X LDS Sample Buffer Blank
2	Test Substance (20 ng)	12	Heat-Treated Control Diet (Treatment 5; Day 0)
3	Test Substance (10 ng)	13	Heat-Treated Control Diet (Treatment 5; Day 3)
4	Test Substance (5 ng)	14	1X LDS Sample Buffer Blank
5	1X LDS Sample Buffer Blank	15	Treatment 2 IPD072Aa Protein Test Dosing Solution (Day 0)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 3)	16	Treatment 2 IPD072Aa Protein Test Dosing Solution (after final use)
7	IPD072Aa Protein Test Diet (Treatment 4; Day 0)	17	Treatment 3 IPD072Aa Protein Test Dosing Solution (Day 0)
8	1X LDS Sample Buffer Blank	18	Treatment 3 IPD072Aa Protein Test Dosing Solution (after final use)
9	Bioassay Control Diet (Treatment 1; Day 0)	19	Treatment 4 IPD072Aa Protein Test Dosing Solution (Day 0)
10	Bioassay Control Diet (Treatment 1; Day 3)	20	Treatment 4 IPD072Aa Protein Test Dosing Solution (after final use)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatment 2 and Treatment 4 were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective test dosing solution.

Figure 37. Dose Confirmation of the IPD072Aa Protein in Treatments 2 and 4, Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5, and Stability of the IPD072Aa Protein in the Test Dosing Solutions

F7.a. Mexican Bean Beetle IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the MBB bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials ASTM Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in two test diets (Treatments 2 and 4; 100 ng and 1000 ng IPD072Aa protein per mg diet wet weight, respectively). Homogeneity assessment of two test diets on one day of diet preparation each is considered representative of both days of diet preparation for all test diets. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively), stability under bioassay conditions of the IPD072Aa protein in Treatment 2, and the stability of the IPD072Aa protein dosing solutions used to prepare Treatments 2, 3, and 4 were also assessed.

Preparation of Solutions

Bulk dosing solutions were prepared on Day 0 of the *MBB* bioassay and maintained chilled (in a refrigerator set at 4 °C or on wet ice) until use. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.133 mg/ml, 0.667 mg/ml, and 1.33 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution for the MBB bioassay, samples were collected shown in Table 145

Table 145. MBB IPD072Aa Protein Bioassay Diet Samples

Treatment	Diet Preparation Day	Number of Samples	Analysis
2	0	5 beginning	Homogeneity of IPD072Aa protein
		5 middle	
		5 end	
4	3	5 beginning	
		5 middle	
		5 end	
4	0	1	Visual confirmation of IPD072Aa protein dose
2	3	NA (bioassay stability sample was used)	
1	0, 3	1 from each treatment each day	Verify presence or absence of immunodetectable IPD072Aa protein
5			
1	3	5 (for Day 0 assessment)	Oven dry weight equivalence
		20 (distributed to bioassay plates)	
2	3	3 (for Day 0 assessment)	Stability under bioassay conditions
		20 (distributed to bioassay plates)	

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, the samples of Treatment 1 and Treatment 2 (20 each) distributed to bioassay plates were placed under bioassay conditions using the same methods and conditions used in the *MBB* bioassay, except they were not infested with larvae. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Days 1-4), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A

combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1, 2, 4, and 5 were weighed to approximately 40 mg on wet ice in preparation for SDS-PAGE. For dose confirmation and assessment of homogeneity in Treatments 2 and 4, Day 0 stability under bioassay conditions in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance and sub-samples of the test dosing solutions (Day 0 and following final use) were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance or test dosing solution, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. The following samples were then loaded into 4-12% Bis-Tris gels as shown in Table 146.

Table 146. MBB IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment	Day(s)	Number of Samples
Dose confirmation of IPD072Aa protein	2	3	1
	4	0	1
Homogeneity	2	0	3 beginning
			3 middle
			3 end
	4	3	3 beginning
			3 middle
			3 end
Stability under bioassay conditions	2	0-4	3 per timepoint
Stability of IPD072Aa protein in test dosing solutions	Used to prepare Treatment 2	Day 0, after final use	1 per timepoint
	Used to prepare Treatment 3		1 per timepoint
	Used to prepare Treatment 4		1 per timepoint
Presence/absence of IPD072Aa protein	1	0, 3	1 per timepoint
	5		1 per timepoint

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for 1 minute each to reduce the background. The blocked membranes were incubated

with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F7.b. Mexican Bean Beetle IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the MBB bioassay was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on Day 0 of the MBB bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 10% Treatment 1 from the MBB bioassay by wet weight of WCR diet)

Treatment B: Test Diet (containing 10% Treatment 4 from the MBB bioassay and targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals

Diet Preparation

Samples were collected from Treatments 1 and 4 on Day 0 and Day 3 diet preparation for the MBB bioassay and were used to prepare Treatments A and B (Day 0 and Day 3 diet preparation) for the WCR bioassay as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the MBB bioassay, resulting in a 10% incorporation of the MBB diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 4 from the MBB bioassay, resulting in a 10% incorporation of the MBB diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 3, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 136.

F8. Evaluation of the Survival and Development of Convergent Lady Beetle Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the CNV bioassay consisted of *Ephestia* (*Ephestia kuehniella* sp.) eggs.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section F8.a. CNV IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was Hippodamia convergens (convergent lady beetle; Coleoptera: Coccinellidae; CNV). CNV was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. CNV larvae were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

The test system for the sensitive insect bioassay was Diabrotica virgifera virgifera (western corn rootworm; Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

CNV larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 15,000 ng boric acid per mg diet dry weight)

Treatments were arranged in a randomized complete block design with a total of 30 blocks. Each block consisted of a stack of six Petri dishes secured together and contained one replicate from each treatment. Each treatment was fed to a target of 30 CNV individuals. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. After 28 days, the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions and the frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the CNV bioassay.

Bias in the CNV bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-5 were prepared and maintained as described in the Analytical Phase section below. The positive control dosing solution for Treatment 6 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 15,000 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 0.75:1 ratio (i.e., 0.75 ml dosing solution to 1 g carrier) to generate Treatments 1-6. After mixing, diets were lyophilized and then pooled together by treatment. The bulk diets were aliquoted into individual storage tubes and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1-5 were collected for characterization of diets as described in the Analytical Phase section below. In addition, a portion each of Treatment 1 and Treatment 4 was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in the Sensitive Insect Bioassay section below.

The positive control diet (Treatment 6) was not characterized.

CNV Bioassay

CNV eggs were incubated in an environmental chamber until the eggs hatched. CNV neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet aliquots were removed from storage and approximately 120 mg of diet were aliquoted into individual small caps. Caps containing the appropriate diet treatment and a moisture source (a tube filled with 0.5% agar) were distributed to each Petri dish utilized in the bioassay. One CNV neonate was placed in each dish and the dishes were stacked into blocks and secured together. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for 28 days. Every 3-4 days, bioassay dishes were removed from the environmental chamber, pupation was assessed, and missing or dead organisms were recorded. For each living

organism that had not yet pupated or died, old diet was removed from the dish and new diet-filled caps were prepared and distributed as described for Day 0, prior to returning the dishes to the environmental chamber. Once pupation was observed, organisms were assessed daily for adult emergence. CNV adults were weighed within approximately 24 hours of emergence. On Day 28, the bioassay was complete and any organisms that had not emerged were considered dead for the purpose of statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality, weight and number of days to adult emergence. Statistical comparisons were made between CNV fed diet containing IPD072Aa (Treatment 2, 3, or 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of CNV fed each of the artificial insect diets containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis tests were

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight was treated as the response and analyzed using the model:

$$y_{ij} = W_i + \varepsilon_{ij}$$

where W_i denotes the mean of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} sample of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$ were independently and identically distributed. This assumption was confirmed by inspection of the residuals from the fitted model.

Each diet containing IPD072Aa protein (w_T) was compared to the bioassay control diet (w_C) to determine if the weight of *CNV* fed the test diet was reduced. The corresponding hypothesis tests were

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Number of Days to Adult Emergence

The distributions of days to adult emergence were evaluated for each treatment. The data were not normally distributed; therefore, non-parametric two-sample tests were conducted separately to examine if exposure to IPD072Aa protein of each concentration caused developmental delays compared to exposure to the bioassay control diet. The corresponding hypothesis tests were

$$H_0 : F_T(x) = F_c(x) \ \ vs. \ H_a : F_T(x) > F_c(x)$$

Where $F_T(x)$ is the cumulative distribution of the number of days to adult emergence of *CNV* fed the artificial diet containing IPD072Aa protein, and $F_c(x)$is the cumulative distribution of the number of days to adult emergence of individuals fed the bioassay control diet. Therefore, significance would indicate that insects fed the diet containing IPD072Aa protein would have a greater probability to take longer to emerge than those fed the control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Demonstration of IPD072Aa Protein Activity in the CNV Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 4 in the *CNV* bioassay. Details regarding the sensitive insect bioassay are provided in Sensitive Insect Bioassay section below.

Results and Discussion

The CNV bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 6) group exceeded 80%, as shown in **Table 147**.

The mortality of CNV fed the test diets containing 100 ng/mg and 500 ng/mg IPD072Aa protein (Treatments 2 and 3, respectively; 0%; P-value = 1.0000) was not significantly greater than those fed the bioassay control diet (Treatment 1; 0%), as shown in Table 147. The mortality of CNV fed the test diet containing 1000 ng/mg IPD072Aa protein (Treatment 4; 56.7%; P-value < 0.0001) was significantly greater than those fed the bioassay control diet (Treatment 1), as shown in Table 147.

The mean weight of newly emerged adult CNV fed Treatments 2, 3, and 4 (18.6 mg, 11.3 mg, and 8.63 mg, P-values = 0.0236, < 0.0001, and < 0.0001, respectively) was significantly less than those fed Treatment 1 (19.7 mg), as shown in Table 148. While there was an observed statistical difference between CNV fed 100 ng/mg IPD072Aa and the control diet, this result is not thought to be biologically significant. Rodriguez-Saona and Miller (1999) showed similar levels of variability in CNV adult weight in experiments assessing maturation differences when reared under four different temperatures. Thus, it is unlikely that an approximately 1 mg difference in mean adult weight is biologically relevant in this context.

CNV fed Treatment 2 (median 14 days; Wilcoxon test P-value 0.0551) and Treatment 1 (median 14 days) did not significantly differ in their probability to take longer to emerge (measured as days to adult emergence) as shown in Table 149. In addition, the Siegel-Tukey test (P-value 0.7594) did not show significant evidence that the scales of the two populations differed. However, Treatments 3 and 4 (median 17 and 22 days, respectively; Wilcoxon test P-values 0.0001, each) both had significantly greater probability to take longer to emerge than those fed Treatment 1. In addition, the Siegel-Tukey test for Treatment 3 (P-value 1.0000) did not show significant evidence that the scales of the populations differed. For Treatment 4, the Siegel-Tukey test (P-value 0.0015) did show significant evidence that the scales of the populations differed.

Western blot analysis visually confirmed the dose and homogeneity of the IPD072Aa protein in Treatments 2-4 (Figure 38, Figure 39, Figure 40, and Figure 41). The stability under bioassay conditions of the IPD072Aa protein in Treatment 2; Figure 42), and frozen storage stability of Treatment 2 (Figure 43), were also assessed. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 were also assessed (Figure 38). The IPD072Aa protein

was detected in the heat-treated control diet (Treatment 5; Figure 38); however, the band was less intense than that of the test diets (Treatments 2, 3, and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 38).

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 4. The WCR bioassay met the acceptability criterion (Appendix B). The biological activity of the IPD072Aa protein in Treatment 4 of the CNV bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in Sensitive Insect Bioassay section below).

The results demonstrated the mortality for CNV fed the test diets containing 100 ng and 500 ng IPD072Aa protein per mg diet (Treatment 2 and Treatment 3, respectively; both 0%) was not significantly greater than the mortality for CNV fed the bioassay control diet (Treatment 1; 0.0%). A statistically significant difference was observed in mortality between CNV fed the test diet containing 1000 ng IPD072Aa protein per mg diet (Treatment 4; 56.7%) and those fed Treatment 1. Statistically significant differences were observed in mean weight between CNV fed Treatments 2, 3 and 4 (18.6 mg, 11.3 mg, and 8.63 mg, respectively) and those fed Treatment 1 (19.7 mg). CNV fed Treatment 2 (median 14 days) and Treatment 1 (median 14 days) did not significantly differ in their probability to take longer to emerge. CNV fed Treatments 3 and 4 (median 17 and 22 days, respectively) had significantly greater probability to take longer to emerge than those fed Treatment 1.

Table 147. Summary Analysis of CNV IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	0	0	1.0000
3	Test Diet	500	30	0	0	1.0000
4	Test Diet	1000	30	17[a]	56.7	<0.0001[b]
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0[c]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight.

[a] Two organisms had not emerged as adults by Day 28 of the bioassay and were scored as dead.

[b] A statistically significant difference (P-value < 0.05) was observed.

[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 148. Summary Analysis of CNV IPD072Aa Protein Bioassay Adult Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	19.7 (18.9 - 20.4)	16.6 - 23.8	--
2	Test Diet	100	30	18.6 (17.9 - 19.3)	11.6 - 22.6	0.0236[a]
3	Test Diet	500	30	11.3 (10.6 - 12.1)	8.9 - 15.2	<0.0001[a]
4	Test Diet	1000	13	8.63 (7.51 - 9.75)	6.9 - 11.7	<0.0001[a]
5	Heat-treated Control Diet	1000	29	18.5 ± 3.63[b]	11.5 - 25.1	--
6	Positive Control Diet	0[c]	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving Hippodamia convergens in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Standard deviation is provided for mean values not subjected to a t-test.

[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 149. Summary Analysis of CNV IPD072Aa Protein Bioassay Results for Days to Adult Emergence

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	30	13.8 ± 0.664	14	13 - 15	--	--
2	Test Diet	100	30	14.1 ± 0.712	14	13 - 16	0.0551	0.7594
3	Test Diet	500	30	17.4 ± 1.63	17	15 - 21	<0.0001[a]	1.0000
4	Test Diet	1000	13	22.3 ± 2.25	22	19 - 25	<0.0001[a]	0.0015[a]
5	Heat-treated Control Diet	1000	29	14.6 ± 1.50	14	13 - 20	--	--
6	Positive Control Diet	0[b]	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving CNV in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

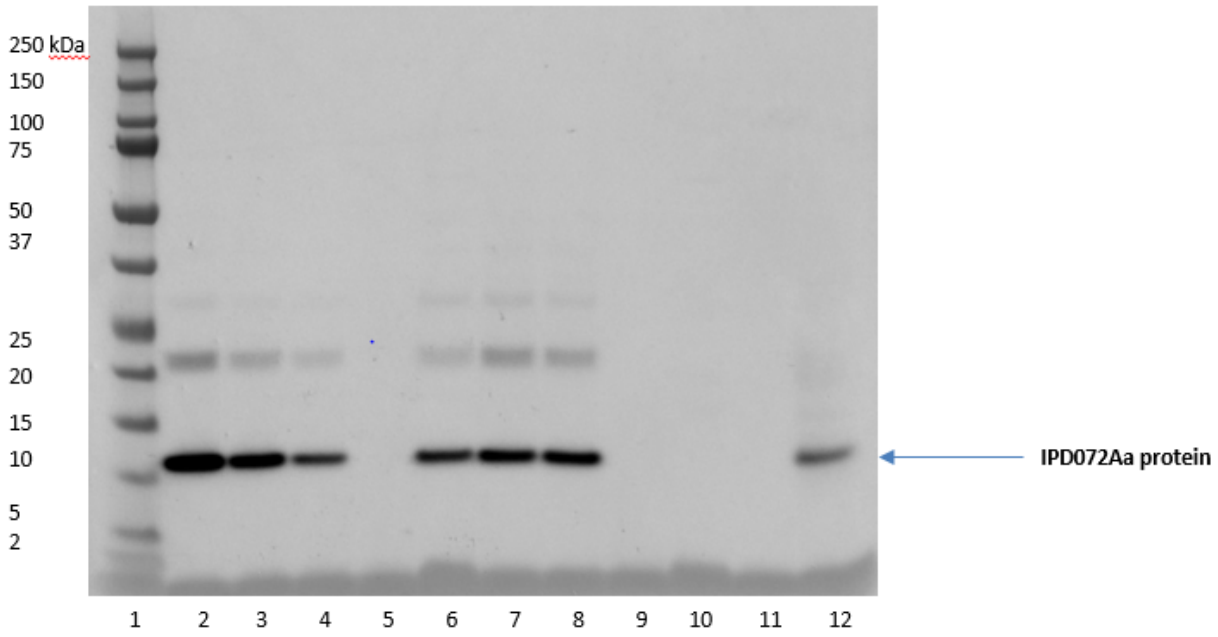
[b] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 150. Summary of CNV IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0 ng IPD072Aa/mg	29[a]	6.90	27	0.415 ± 0.149	0.1 - 0.7
B	Test Diet	85.5 ng IPD072Aa/mg	26[a]	92.3	2	0.300 ± 0.283	0.1 - 0.5

Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 4, respectively, of the CNV bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

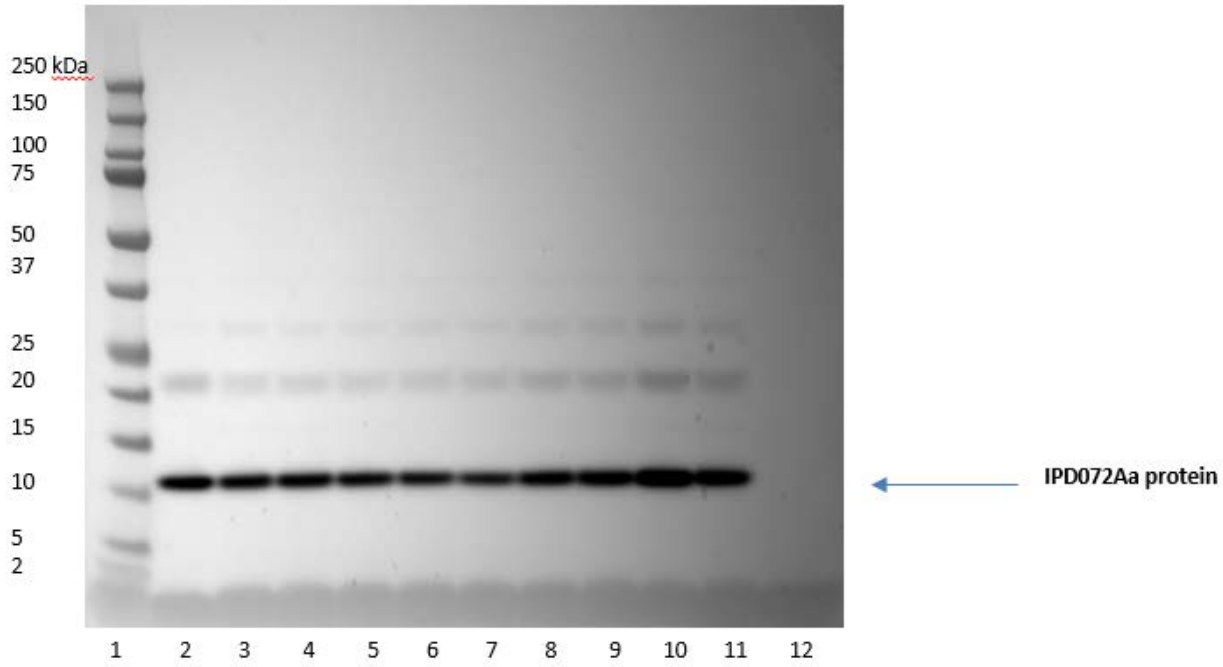
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2)
7	IPD072Aa Protein Test Diet (Treatment 3)
8	IPD072Aa Protein Test Diet (Treatment 4)
9	1X LDS Sample Buffer Blank
10	Bioassay Control Diet (Treatment 1)
11	1X LDS Sample Buffer Blank
12	Heat-Treated Control Diet (Treatment 5)

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatment 2, 3, and 4 were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

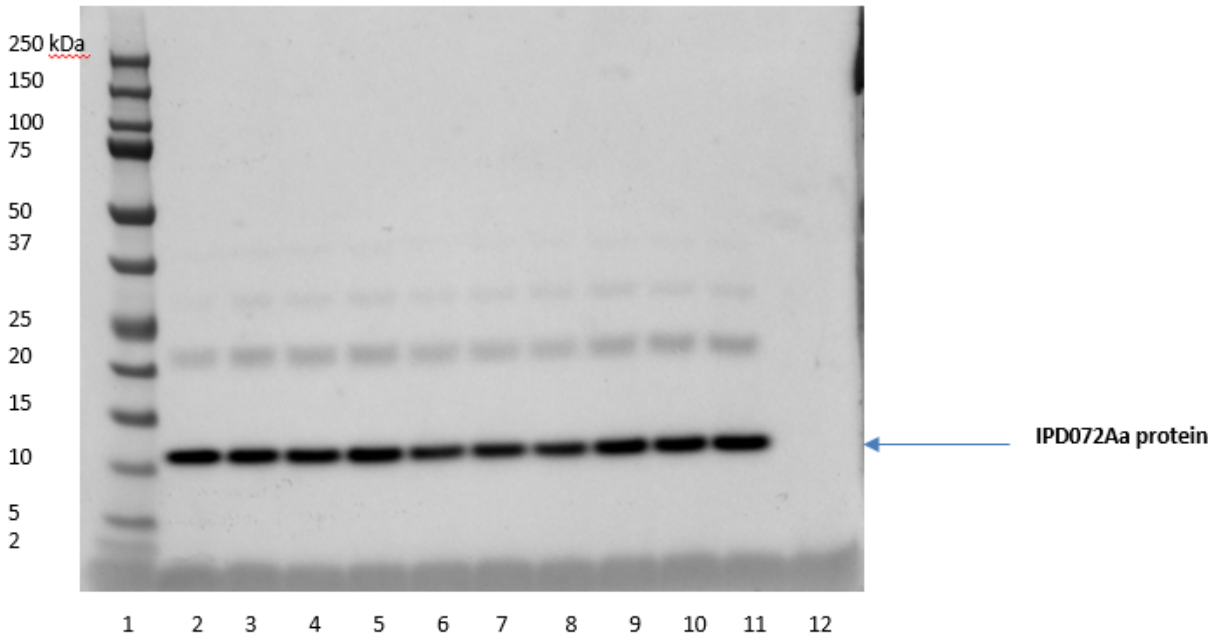
Figure 38. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3, and 4 and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

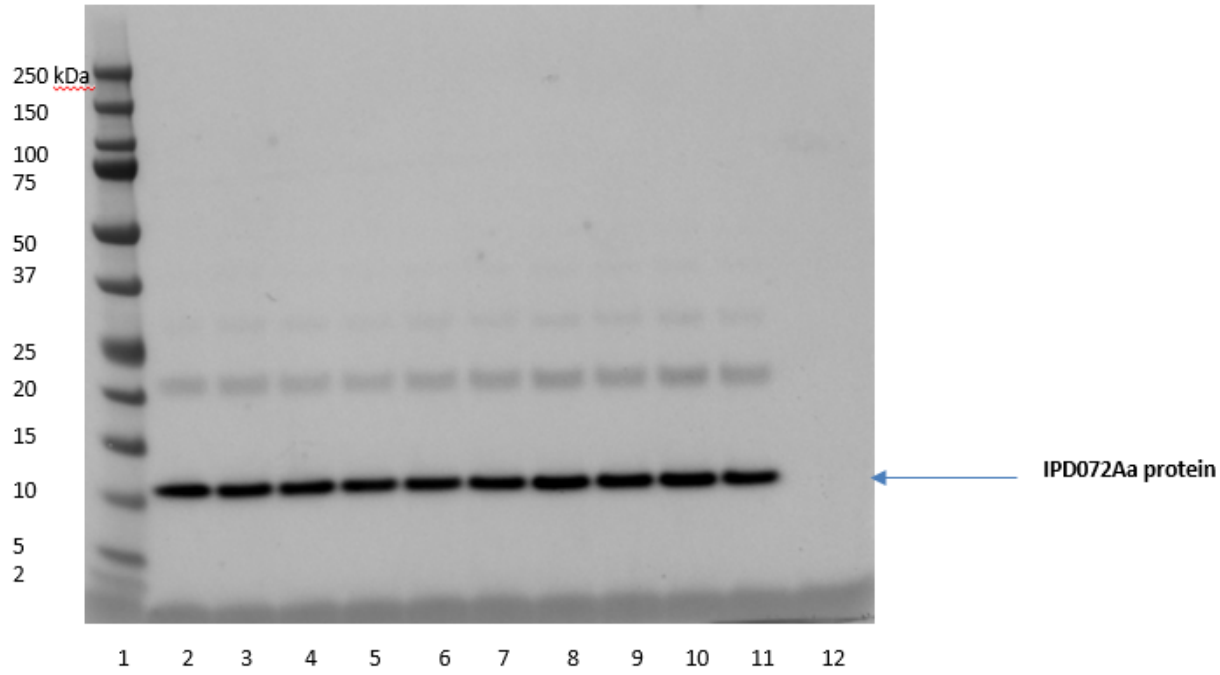
Figure 39. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; End)
10	IPD072Aa Protein Test Diet (Treatment 3; End)
11	IPD072Aa Protein Test Diet (Treatment 3; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein Treatment 3.

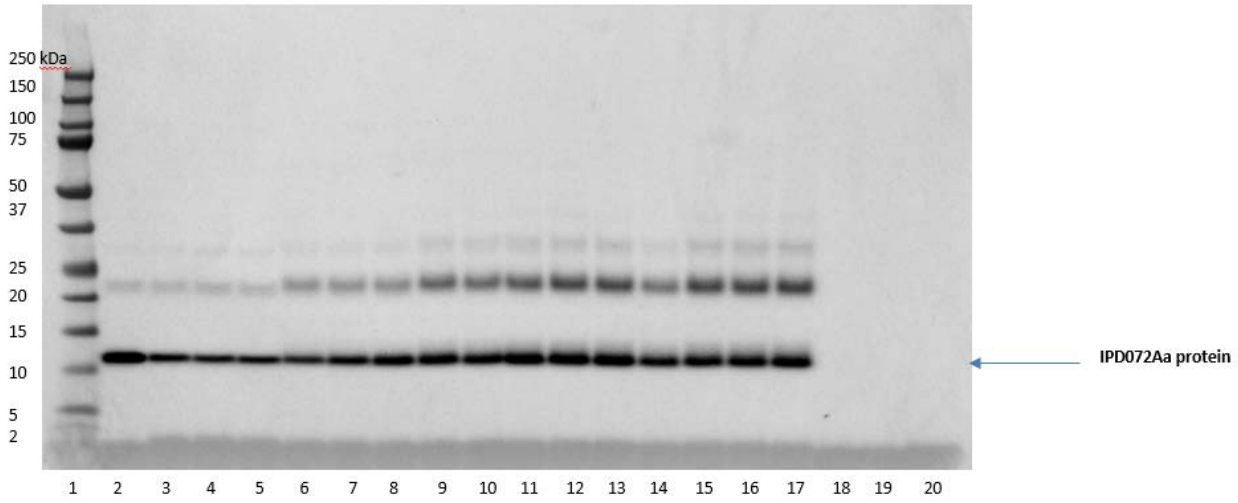
Figure 40. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

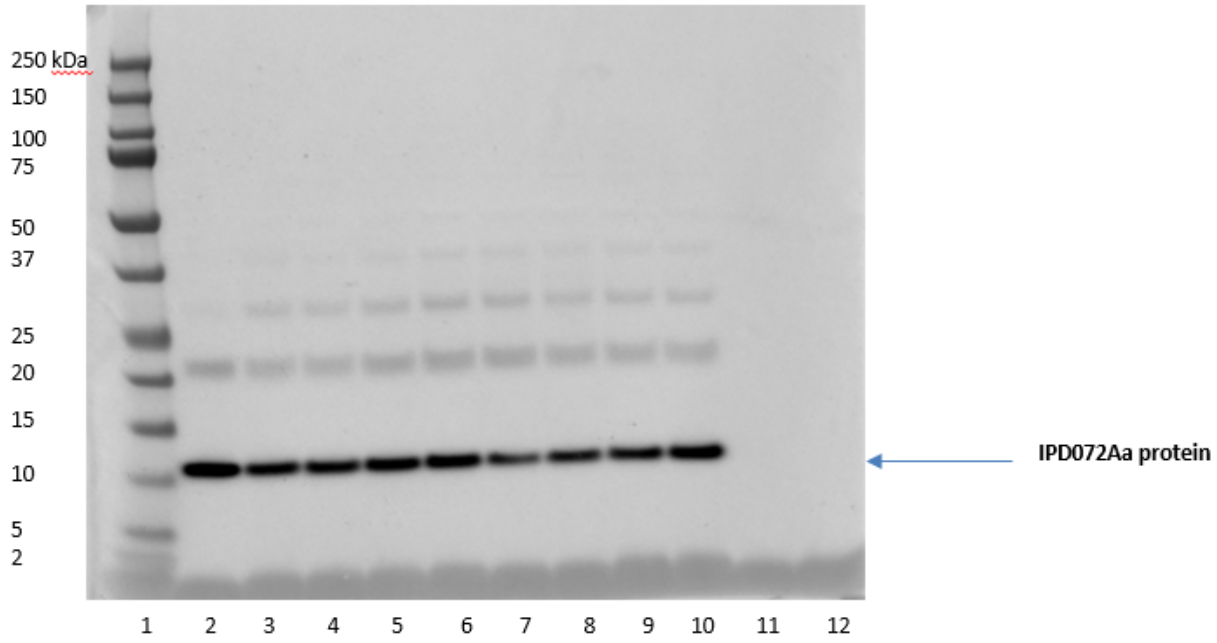
Figure 41. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
2	Test Substance (10 ng)	12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	18	1X LDS Sample Buffer Blank
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	19	1X LDS Sample Buffer Blank
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	20	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Days 0-4 refer to the number of days for which samples were stored under bioassay conditions prior to analysis.

Figure 42. Stability Assessment of the IPD072Aa Protein in Treatment 2 under Bioassay Conditions



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
6	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
9	IPD072Aa Protein Test Diet (Treatment 2; Week 6)
10	IPD072Aa Protein Test Diet (Treatment 2; Week 6)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Week 1, Week 4, and Week 6 refer to the approximate length of time samples were stored frozen (-80°C freezer unit) prior to analysis.

Figure 43. Frozen Storage Stability Assessment of the IPD072Aa Protein in Treatment 2

F8.a. CNV IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the CNV bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of IPD072Aa protein test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in three test diets (Treatments 2, 3, and 4; 100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet dry weight, respectively). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively), stability under bioassay conditions and frozen storage stability of the IPD072Aa protein in Treatment 2 were also assessed.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.133 mg/ml, 0.667 mg/ml, and 1.333 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the CNV bioassay, samples were collected as shown in Table 151.

Table 151. CNV IPD072Aa Protein Bioassay Diet Samples

Treatment	Number of Samples	Analysis
2	5 beginning	Homogeneity of IPD072Aa protein
	5 middle	
	5 end	
3	5 beginning	
	5 middle	
	5 end	
4	5 beginning	
	5 middle	
	5 end	
2	NA (One homogeneity sample was used from each treatment)	Visual confirmation of IPD072Aa protein dose
3		
4		
1	1 from each treatment	Verify presence or absence of immunodetectable IPD072Aa protein
5		
1	5 (for Day 0 assessment)	Oven dry weight equivalence
	20 (distributed to dishes)	
2	NA (3 homogeneity samples were used for Day 0 assessment)	Stability under bioassay conditions
	20 (distributed to Petri dishes)	
2	NA (2 homogeneity samples were used for Day 0 assessment)	Frozen storage stability
	2 (for each timepoint; Week 1, Week 4, and Week 6)	

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, the aliquots of Treatment 1 and Treatment 2 were removed from storage, distributed to dishes (20 for each treatment), and placed under bioassay conditions using the same methods and conditions used in the *CNV* bioassay, except they were not infested with larvae. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Days 1-4), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1, 2, 3, 4, and 5 were weighed to approximately 10 mg on dry ice in preparation for SDS-PAGE. For dose confirmation and assessment of homogeneity in Treatments 2, 3, and 4, Day 0 stability under bioassay conditions and storage stability in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown in Table 152.

Table 152. CNV IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment	Number of Samples
Dose confirmation of IPD072Aa protein	2, 3, and 4	1 per treatment
Homogeneity	2	3 beginning
		3 middle
		3 end
	3	3 beginning
		3 middle
		3 end
	4	3 beginning
		3 middle
		3 end
Stability under bioassay conditions	2	3 per timepoint (Days 0-4)
Storage Stability	2	2 per timepoint (Day 0, Weeks 1, 4, and 6)
Presence/absence of IPD072Aa protein	1	1
	5	1

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred

International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for at least 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for at least 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F8.b. CNV IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the CNV bioassay was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after the CNV bioassay was complete.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)

Treatment B: Test Diet (containing a portion of Treatment 4 and targeting 85.5 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 4 during diet preparation for the CNV bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the CNV bioassay, resulting in a 30% incorporation of the CNV diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 4 from the CNV bioassay, resulting in a 30% incorporation of the CNV diet by dry weight of the WCR diet.

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100 and

are presented inTable 150 Weight data were summarized as means, standard deviations, and ranges and are presented in Table 150.

F9. Evaluation of the Survival and Development of Pink Spotted Lady Beetle Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the CMAC bioassay consisted of decapsulated brine shrimp (*Artemia* sp.) eggs. The carrier for the sensitive insect WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Positive Control

The positive control consisted of cryolite (AlF_6Na_3).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section F9.a. Pink Spotted Lady Beetle IPD072Aa Bioassay Analytical Phase.

Test System

The test system was CMAC (pink spotted lady beetle; Coleoptera: Coccinellidae). CMAC was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa

protein. CMAC eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay wasWCR. The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

CMAC larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 10,000 ng cryolite per mg diet dry weight)

Treatments were arranged in a randomized complete block design with a total of 30 blocks. Each block consisted of a stack of six Petri dishes secured together and contained one replicate from each treatment. Each treatment was fed to a target of 30 CMAC individuals. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photometric cycle of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. On Day 23, all organisms had either died or emerged and the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions and the storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the CMAC bioassay.

Bias in the CMAC bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-5 were prepared and maintained as described in the Analytical Phase section below. Each dosing solution was mixed with carrier in a 1:1 ratio (i.e., 1 g carrier to 1 ml dosing solution). Treatment 6 was prepared by mixing cryolite with carrier to a nominal concentration of 10,000 ppm by carrier dry weight and then combining with ultrapure water in a 1:1 ratio. After mixing, diets were transferred to 50-ml tubes, lyophilized, and then pooled together by treatment. The bulk diets were aliquoted into individual storage tubes and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1-5 were collected for characterization of diets as described in Analytical Phase section below. The positive control diet (Treatment 6) was not characterized. In addition, a portion each of Treatment 1 and Treatment 4 was collected during the process of diet aliquoting for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in the Sensitive Insect Bioassay section below.

CMAC Bioassay

A bioassay was conducted to determine the response of CMAC to IPD072Aa protein exposure via oral ingestion. CMAC eggs were incubated in an environmental chamber until the eggs hatched. CMAC neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet aliquots were removed from storage and approximately 20 mg of diet were aliquoted into individual caps. A cap containing the appropriate diet treatment and a moisture source (a tube filled with 0.5% agar) were distributed to each Petri dish utilized in the bioassay. One CMAC neonate was placed in each dish and the dishes were stacked into blocks and secured together. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photometric cycle of 16 hours of light followed by 8 hours of dark. Every 3-4 days, bioassay dishes were removed from the environmental chamber, pupation was assessed, and missing or dead organisms were recorded. For each living organism that had not yet pupated or died, old diet was removed from the dish and a new diet-filled cap was prepared and distributed as described for Day 0, prior to returning the dishes to the environmental chamber. Once pupation was observed, organisms were assessed daily for adult emergence. CMAC adults were weighed within approximately 24 hours of emergence. The bioassay was ended when all larvae had either died or emerged as adults and final mortality was assessed. Only dishes that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a dish were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality, weight and number of days to adult emergence. Statistical comparisons were made between CMAC fed diet containing IPD072Aa protein (Treatment 2, 3, or 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

CMAC fed each of the artificial insect diets containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis tests were

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight was treated as the response and analyzed using the model:

$$y_{ij} = W_i + \varepsilon_{ij}$$

where W_i denotes the mean of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} sample of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$ were independently and identically distributed. This assumption was confirmed by inspection of the residuals from the fitted model.

Each diet containing IPD072Aa protein (w_T) was compared to the bioassay control diet (w_C) to determine if the weight of CMAC fed the test diet was reduced. The corresponding hypothesis tests were

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis, and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Number of Days to Adult Emergence

The distributions of days to adult emergence were evaluated for each treatment. The data were not normally distributed; therefore, non-parametric two-sample tests were conducted separately to examine if exposure to IPD072Aa protein of each concentration caused developmental delays compared to exposure to the bioassay control diet. The corresponding hypothesis tests were

$$H_0 : F_T(x) = F_c(x) \ \ vs. \ H_a : F_T(x) > F_c(x)$$

Where $F_T(x)$ is the cumulative distribution of the number of days to adult emergence of *CMAC* fed the artificial insect diet containing IPD072Aa protein, and $F_c(x)$ is the cumulative distribution of the number of days to adult emergence of individuals fed the bioassay control diet. Therefore, significance would indicate that insects fed the diet containing IPD072Aa protein would have a greater probability to take longer to emerge than those fed the control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Demonstration of IPD072Aa Protein Activity in the CMAC Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 4 in the CMAC bioassay. Details regarding the sensitive insect bioassay are provided in Sensitive Insect Bioassay section below.

Results and Discussion

The CMAC bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 153.

The mortality of CMAC fed the test diet containing 100 ng/mg IPD072Aa protein (Treatment 2; 6.67% mortality; P-value = 0.7070) was not significantly greater than those fed the bioassay control diet (Treatment 1; 6.90% mortality), as shown in Table 153. The mortality of CMAC fed the test diets containing 500 ng/mg and 1000 ng/mg IPD072Aa protein (Treatments 3 and 4; 26.7% and 36.7% mortality, P-values 0.0449 and 0.0061, respectively) was significantly greater than those fed Treatment 1 (6.90% mortality), as shown in Table 153.

The mean weight of CMAC fed Treatment 2 (12.1 mg; P-value = 0.5424) was not significantly less than those fed Treatment 1 (12.0 mg), as shown in Table 154. The mean weight of CMAC fed Treatments 3 and 4 (10.7 and 10.4 mg; P-values 0.0073 and 0.0014, respectively) was significantly less than those fed Treatment 1 (12.0 mg), as shown in Table 154.

CMAC fed Treatment 2 (median 15 days; Wilcoxon test P-value 0.1529) and Treatment 1 (median 14 days) did not significantly differ in the probability to take longer to emerge as shown in Table 3. In addition, the Siegel-Tukey test (P-value 0.6183) did not show significant evidence that the scales of the two populations differed. CMAC fed Treatments 3 and 4 (median 16 days each; Wilcoxon test P-values 0.0015 and 0.0088, respectively) had significantly greater probability to take longer to emerge than those fed Treatment 1. In addition, the Siegel-Tukey test (P-values 0.3759 and 0.1147, respectively) did not show significant evidence that the scales of the populations differed.

Western blot analysis visually confirmed the dose and the homogeneity of the IPD072Aa protein in Treatments 2-4 (Figure 44, Figure 45, Figure 46, and Figure 47). The storage stability of Treatment 2 (Figure 48) and the stability under bioassay conditions of the IPD072Aa protein in Treatment 2 (Figure 49), were also visually confirmed by Western blot analysis. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 were also visually confirmed by Western blot analysis (Figure 44). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 44); however, the band was less intense than that of the test diets (Treatments 2, 3, and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 44).

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 156. The WCR bioassay met the acceptability criterion (section F9.a. Pink Spotted Lady Beetle IPD072Aa Protein Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in Treatment 4 of the CMAC bioassay was demonstrated by 100% mortality of WCR fed the test diet (Treatment B; described in section F9.a. Pink Spotted Lady Beetle IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated the mortality for CMAC fed the test diet containing 100 ng IPD072Aa protein per mg diet (Treatment 2; 6.67%) was not significantly greater than the mortality for CMAC fed the bioassay control diet (Treatment 1; 6.90%). Statistically significant differences were observed in mortality between CMAC fed the test diets containing 500 ng and 1000 ng IPD072Aa protein per mg diet (Treatment 3 and 4; 26.7% and 36.7%, respectively) and those fed Treatment 1 (6.90%). The mean weight for CMAC fed Treatment 2 (12.1 mg) was not significantly less than the mean weight for CMAC fed Treatment 1 (12.0 mg). Statistically significant differences were observed in mean weight between CMAC fed Treatments 3 and 4 (10.7 mg and 10.4 mg, respectively) and those fed Treatment 1 (12.0 mg). CMAC fed Treatment 2 (median 15 days) and Treatment 1 (median 14 days) did not significantly differ in their probability to take longer to emerge. CMAC fed Treatments 3 and 4 (median 16 days) had significantly greater probability to emerge later than those fed Treatment 1.

Table 153. Summary Analysis of CMAC IPD072Aa Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	29[a]	2	6.90	--
2	Test Diet	100	30	2	6.67	0.7070
3	Test Diet	500	30	8	26.7	0.0449[b]
4	Test Diet	1000	30	11	36.7	0.0061[b]
5	Heat-treated Control Diet	1000	29[a]	3	10.3	--
6	Positive Control Diet	0	30	30	100	--.

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight. Treatment 6 contained a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

Table 154. Summary Analysis of CMAC IPD072Aa Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	27	12.0 (11.3 - 12.7)	5.4 - 14.9	--
2	Test Diet	100	28	12.1 (11.4 - 12.8)	9.0 - 15.7	0.5424
3	Test Diet	500	22	10.7 (9.96 - 11.5)	6.1 - 14.1	0.0073[a]
4	Test Diet	1000	19	10.4 (9.53 - 11.2)	8.2 - 12.3	0.0014[a]
5	Heat-treated Control Diet	1000	26	11.3 ± 1.63[b]	9.6 - 15.0	--
6	Positive Control Diet	0	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Treatment 6 contains a targeted concentration of 10,000 ng cryolite per mg diet dry weight.

[a] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

[b] Standard deviation is provided for mean values not subjected to linear mixed model analysis.

Table 155. Summary Analysis of CMAC IPD072Aa Bioassay Days to Adult Emergence Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean ± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	27	14.6 ± 1.95	14	11 - 22	--	--
2	Test Diet	100	28	14.7 ± 1.18	15	13 - 17	0.1529	0.6183
3	Test Diet	500	22	16.6 ± 2.92	16	13 - 23	0.0015[a]	0.3759
4	Test Diet	1000	19	15.9 ± 2.05	16	13 - 20	0.0088[a]	0.1147
5	Heat-treated Control Diet	1000	26	14.3 ± 1.02	14.5	13 - 16	--	--
6	Positive Control Diet	0	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Treatment 6 contains a targeted concentration of 10,000 ng cryolite per mg diet dry weight. Not applicable (NA); there were no surviving *CMAC* in the positive control diet group.

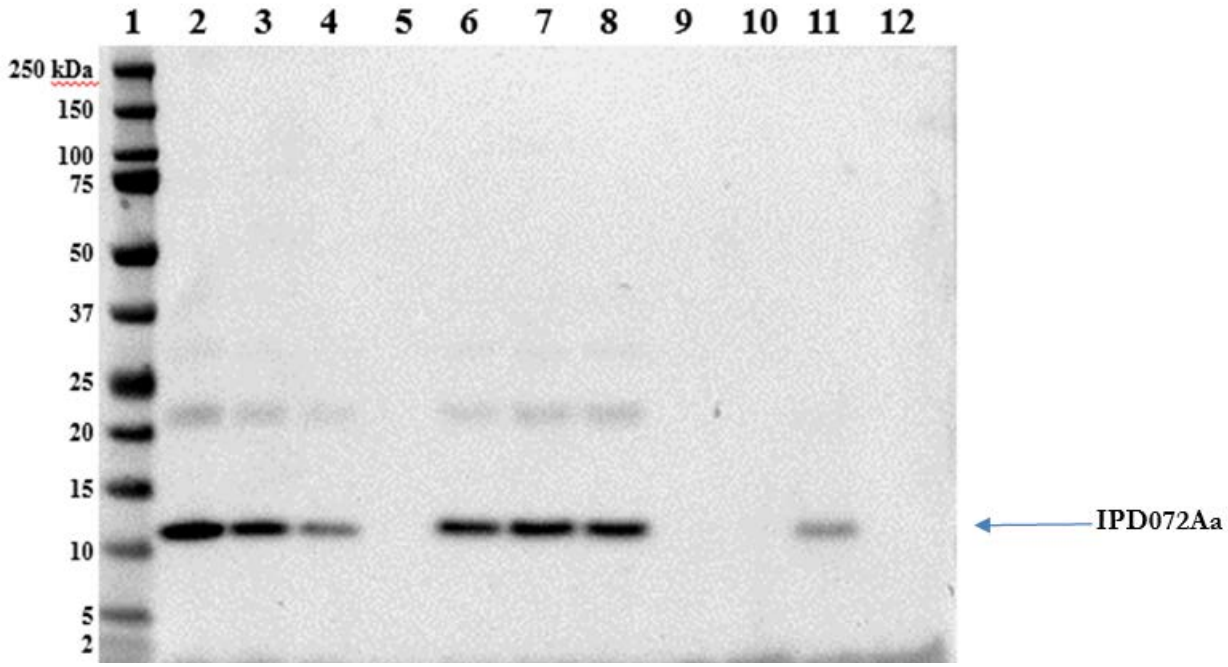
[a] A statistically significant difference (P-value < 0.05) was observed in this treatment compared to Treatment 1.

Table 156. Summary of CMAC IPD072Aa Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0	30	0	0
B	Test Diet	85.5	27[a]	27	100

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera)* bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 4, respectively, of the *CMAC* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

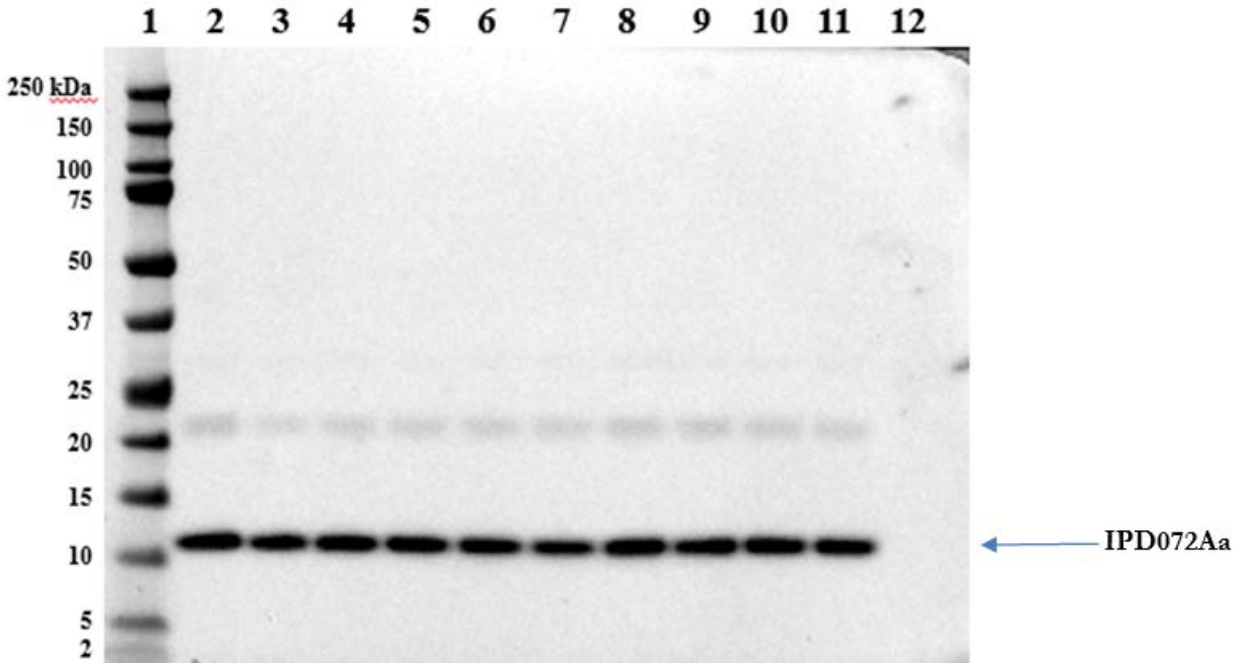
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2)
7	IPD072Aa Protein Test Diet (Treatment 3)
8	IPD072Aa Protein Test Diet (Treatment 4)
9	1X LDS Sample Buffer Blank
10	Bioassay Control Diet (Treatment 1)
11	Heat-Treated Control Diet (Treatment 5)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded undiluted and contained the highest sample matrix concentration. Treatment 2, Treatment 3, and Treatment 4 were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 sample was loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein.

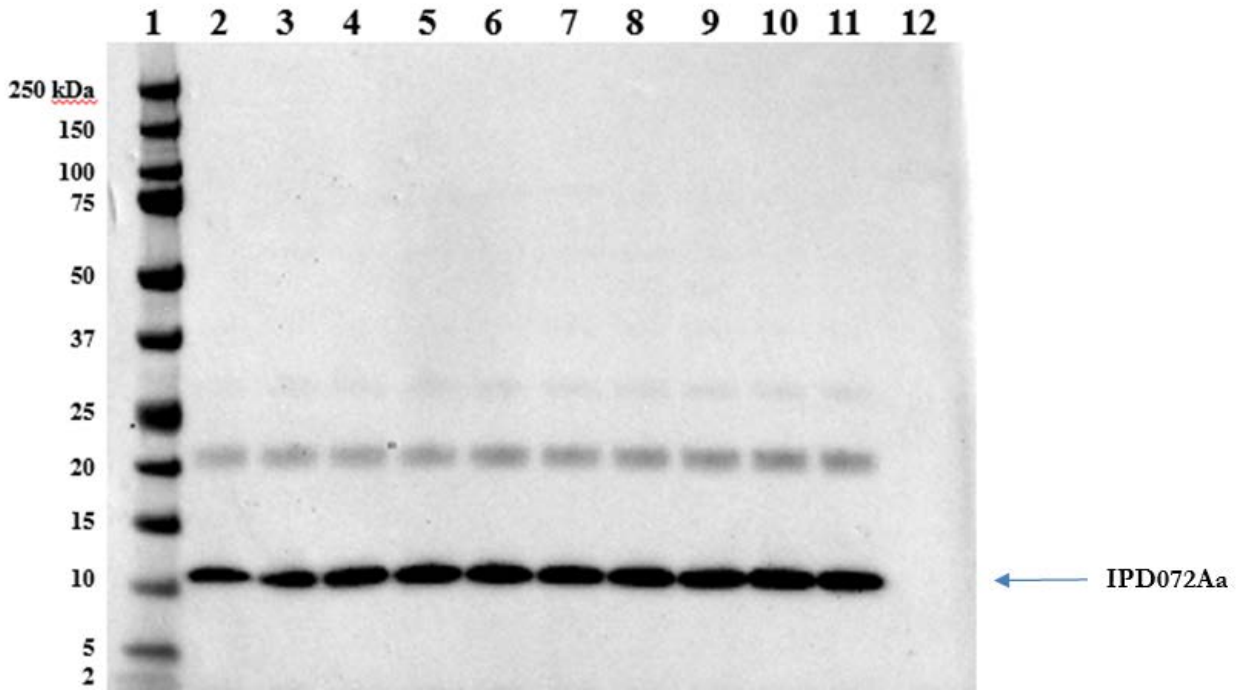
Figure 44. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3, and 4, and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

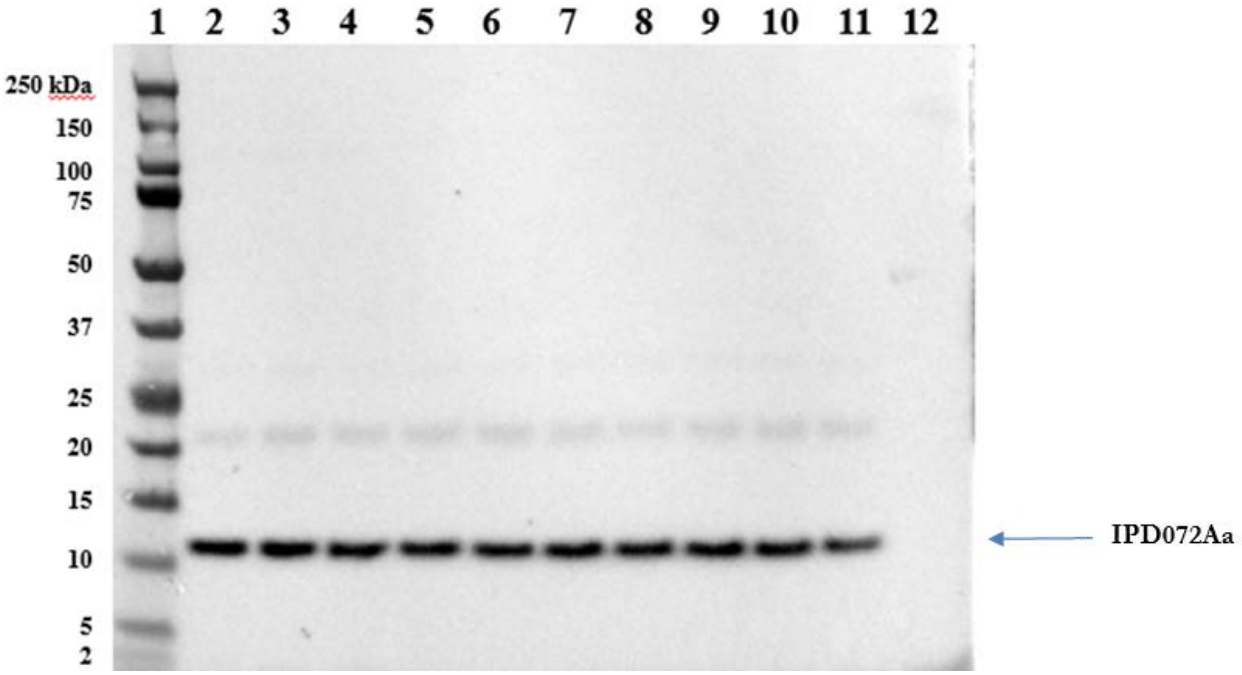
Figure 45. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; End)
10	IPD072Aa Protein Test Diet (Treatment 3; End)
11	IPD072Aa Protein Test Diet (Treatment 3; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 3. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

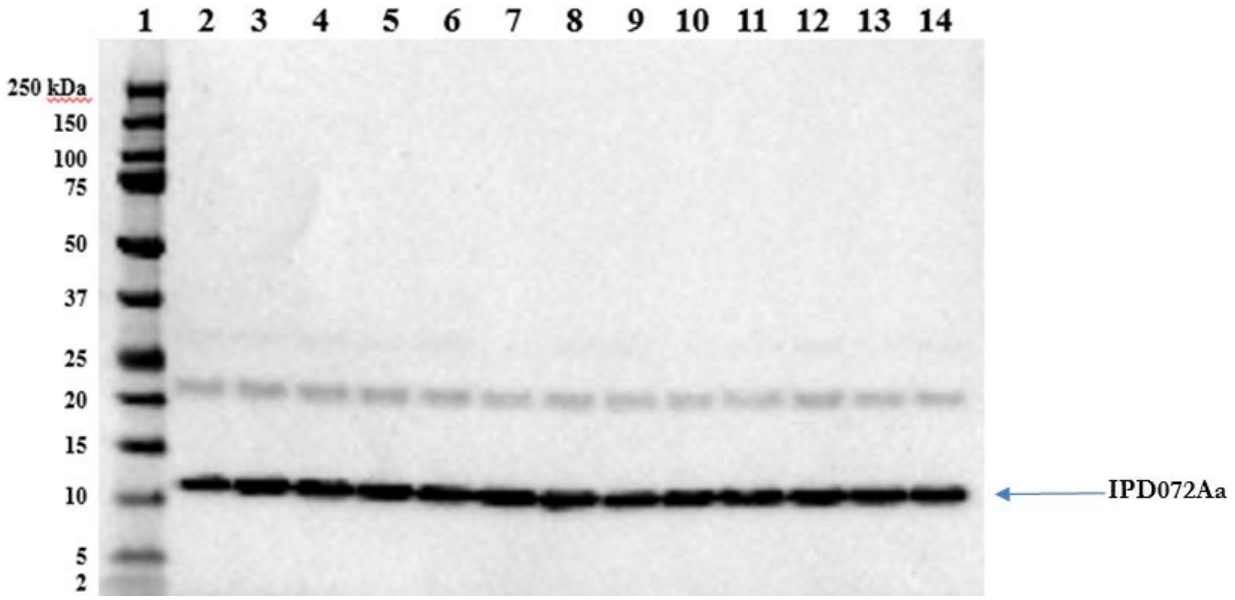
Figure 46. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4 Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

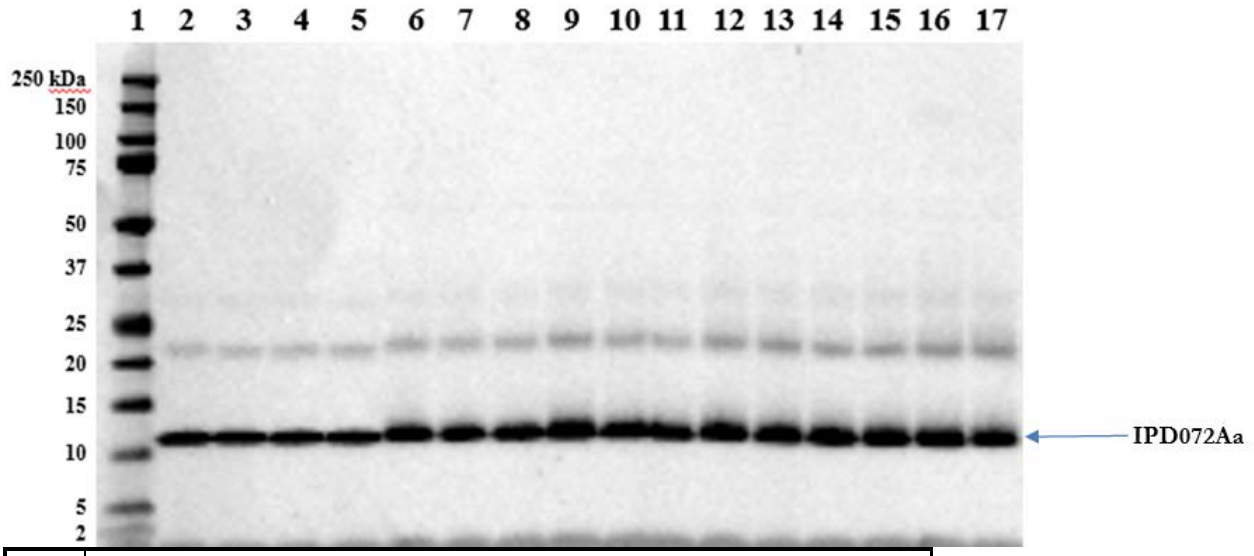
Figure 47. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
6	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Week 2)
8	IPD072Aa Protein Test Diet (Treatment 2; Week 2)
9	IPD072Aa Protein Test Diet (Treatment 2; Week 3)
10	IPD072Aa Protein Test Diet (Treatment 2; Week 3)
11	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
12	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
13	IPD072Aa Protein Test Diet (Treatment 2; Week 5)
14	IPD072Aa Protein Test Diet (Treatment 2; Week 5)

Note: Nanogram (ng). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein Treatment 2. Week 1 through Week 5 refer to the approximate length of time samples were stored frozen (-80°C freezer unit) prior to analysis.

Figure 48. Storage Stability Assessment of the IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Days 0-4 refer to the number of days for which samples were stored under bioassay conditions prior to analysis.

Figure 49. Stability Assessment of the IPD072Aa Protein in Treatment 2 under Bioassay Conditions

F9.a. Pink Spotted Lady Beetle IPD072Aa Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the CMAC (CMAC) bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of IPD072Aa protein test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in three test diets (Treatments 2, 3, and 4; 100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet dry weight, respectively). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively) and the stability under bioassay conditions and storage stability of the IPD072Aa protein in Treatment 2 were also assessed.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.1 mg/ml, 0.5 mg/ml, and 1 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the CMAC bioassay, samples of Treatments 1-5 were collected as shown in **Table 157**

Table 157. CMAC IPD072Aa Bioassay Diet Samples

Treatment	Number of Samples	Analysis
2	5 beginning	Homogeneity of IPD072Aa protein
	5 middle	
	5 end	
3	5 beginning	
	5 middle	
	5 end	
4	5 beginning	
	5 middle	
	5 end	
2	NA (homogeneity sample was used)	Visual confirmation of IPD072Aa protein dose
3	NA (homogeneity sample was used)	
4	NA (homogeneity sample was used)	
1	1 from each treatment	Verify presence or absence of immunodetectable IPD072Aa protein
5		
1	5 (for Day 0 assessment)	Oven dry weight equivalence
	20 (distributed to petri dishes)	
2	3 (for Day 0 assessment; homogeneity samples were used)	Stability under bioassay conditions
	20 (distributed to petri dishes)	
2	2 (for Day 0 assessment; homogeneity samples were used)	Storage Stability
	2 for each week (Week 1 - Week 5)	

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, aliquots of Treatment 1 and Treatment 2 were removed from storage, distributed to dishes (20 for each treatment) and placed under bioassay conditions using the same methods and conditions used in the *CMAC* bioassay, except they were not infested with larvae. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Days 1-4), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A

combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1, 2, 3, 4, and 5 were weighed to approximately 10 mg on dry ice in preparation for SDS-PAGE. For dose confirmation and assessment of homogeneity in Treatments 2, 3, and 4, Day 0 stability under bioassay conditions and storage stability in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3-5 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown in Table 158.

Table 158. CMAC IPD072Aa Bioassay SDS-PAGE Samples

Gel	Treatment	Number of Samples
Dose confirmation of IPD072Aa protein	2	1
	3	1
	4	1
Homogeneity	2	3 beginning
		3 middle
		3 end
	3	3 beginning
		3 middle
		3 end
	4	3 beginning
		3 middle
		3 end
Stability under bioassay conditions	2	3 per timepoint (Days 0-4)
Storage Stability	2	2 per timepoint (Day 0, Week 1-5)
Presence/absence of IPD072Aa protein	1	1
	5	1

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before and after the blocking step, the membranes were washed with PBST to reduce the background. The blocked membranes were incubated with an

IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST three times for at least 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:20,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST three times for at least 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa.

Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F9.a. Pink Spotted Lady Beetle IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the CMAC bioassay was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after all CMAC were observed dead or pupated.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 30% Treatment 1 from the CMAC bioassay by dry weight of WCR diet.

Treatment B: Test Diet (containing 30% Treatment 4 from the CMAC bioassay by dry weight of WCR diet and targeting 85.5 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 4 during diet preparation for the CMAC bioassay were removed from the freezer and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the CMAC bioassay, resulting in a 30% incorporation of the CMAC diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 4 from the CMAC bioassay, resulting in a 30% incorporation of the CMAC diet by dry weight of the WCR diet.

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 156.

F10. Evaluation of the Survival and Weight of Mealybug Destroyer Fed Artificial Diets Containing Purified IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of *Ephestia* (*Ephestia kuehniella* sp.) eggs.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in the Analytical Phase section below.

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was *Cryptolaemus montrouzieri* (mealybug destroyer; Coleoptera: Coccinellidae; MBD). MBD was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. MBD adults were obtained from Beneficial Insectary (Redding, CA, USA) and identity was recorded by study personnel.

The test system for the sensitive insect bioassay was WCR. The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

MBD adults were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 37,500 ng boric acid per mg diet dry weight)

Treatments were arranged in a randomized complete block design with a total of 30 blocks. Each block consisted of a stack of six Petri dishes secured together and contained one replicate from each treatment. Each treatment was fed to a target of 30 MBD individuals and organisms were weighed prior to infestation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Adults were refed every 3 days. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatment 2, 3, and 4 and the stability under bioassay conditions and the frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 4 of the MBD bioassay.

Bias in the MBD bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-5 were prepared and maintained as described in Analytical Phase section below. The positive control dosing solution for Treatment 6 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 37,500 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 0.75:1 ratio (i.e., 0.75 ml dosing solution to 1 g carrier) to generate Treatments 1-6. After mixing, diets were lyophilized, aliquoted into individual storage tubes, and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1-5 were collected for characterization as described in Analytical Phase section below. In addition, a portion each of Treatment 1 and Treatment 4 was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in the Sensitive Insect Bioassay section below.

The positive control diet (Treatment 6) was not characterized.

MBD Bioassay

A bioassay was conducted to determine the response of MBD to IPD072Aa protein exposure via oral ingestion.

On Day 0, diet aliquots of each treatment were removed from storage and approximately 60 mg of diet were dispensed into individual small caps. A cap containing the appropriate diet treatment and a moisture source (a tube filled with 0.5% agar) were distributed to each Petri dish utilized in the bioassay. MBD adults were weighed and one MBD adult was placed in each dish. The dishes were stacked into blocks and secured together. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for a total of 7 days. Every 3 days, bioassay dishes were removed from the environmental chamber and missing or dead organisms were recorded.

Old diet was removed from the dishes and new diet-filled caps were prepared and distributed as described for Day 0, prior to returning the dishes to the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight change. Statistical comparisons were made between MBD fed diet containing IPD072Aa protein (Treatments 2, 3, and 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

MBD fed the artificial insect diets containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was established identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight Change

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight at Day 7 was treated as the response and analyzed using this model:

$$y_{ij} = W_i + \beta x_{ij} + \varepsilon_{ij}$$

Where x_{ij} denotes weight at day 0 of the j^{th} insect of the i^{th} treatment (covariate), W_i denotes the mean change of weight of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} insect of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$ were independently and identically distributed. This assumption was confirmed by visual assessment of plots of residuals.

To test if the weight change of MBD fed the artificial insect diets containing IPD072Aa protein (w_T) was less than the weight change of those fed the bioassay control diet (w_C), the corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis, and the statistical comparison between treatment means.

Demonstration of IPD072Aa Protein Activity in a *MBD* Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 4 in the MBD bioassay. A portion of Treatments 1 and 4 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section below.

Results and Discussion

The MBD bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 6) group exceeded 80%, as shown in Table 153.

The mortality of MBD fed the test diets containing 100 ng/mg, 500 ng/mg, and 1000 ng/mg IPD072Aa protein (Treatments 2, 3, and 4, respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1), as shown in Table 153. Mortality was 26.7% in Treatment 2 (P-value = 0.3805), 13.3% in Treatment 3 (P-value = 0.8505), and 36.7% in Treatment 4 (P-value = 0.1258). Mortality was 20.0% in Treatment 1.

The weight gain from Day 0 to Day 7 for MBD fed Treatment 3 and Treatment 4 (increase in mean weight from 9.68 mg to 12.5 mg and from 9.48 mg to 12.5 mg; P-values= 0.1667 and 0.4681, respectively) was not significantly less than the weight gain from Day 0 to Day 7 for MBD fed Treatment 1 (increase in mean weight from 9.38 mg to 12.4 mg). A statistically significant difference was observed in the weight gain from Day 0 to Day 7 between MBD fed Treatment 2 (increase in mean weight from 9.66 mg to 12.2 mg; P-value = 0.0252) and those fed Treatment 1.

Western blot analysis visually confirmed the dose and the homogeneity of the IPD072Aa protein in Treatments 2-4 (Figure 44, Figure 45, Figure 46, and Figure 47). The stability under

bioassay conditions of the IPD072Aa protein in Treatment 2 (Figure 48), and frozen storage stability of Treatment 2 (Figure 43), were also assessed. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 were also assessed (Figure 44). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 44); however, the band was less intense than that of the test diets (Treatments 2, 3, and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 44).

The WCR bioassay met the acceptability criterion (Sensitive Insect Bioassay section). The biological activity of the IPD072Aa protein in Treatment 4 of the MBD bioassay was demonstrated by 100% mortality of WCR fed the test diet (Treatment B; described in the Sensitive Insect Bioassay section). Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 155.

Conclusion

No statistically significant differences in mortality were observed in MBD exposed to a concentration of 100, 500 or 1000 ng IPD072Aa protein per mg diet when compared to those fed the bioassay control diet. While survival differences were not statistically significant, MBD fed 100 and 1000 ng IPD072Aa protein per mg diet exceeded 20% mortality. Development (measured as weight gain over the 7-day bioassay) was statistically significantly lower in MBD fed 100 ng IPD072Aa protein per mg diet versus the bioassay control, but not with those fed 500 and 1000 ng IPD072Aa protein per mg diet. In the absence of a dose-dependent response, the statistically significant difference for 100 ng IPD072Aa protein per mg diet is not thought to be biologically relevant. It can be concluded that at concentrations up to 1000 ng IPD072Aa protein per mg diet, there was no effect on survival or weight gain of MBD.

Table 159. Summary Analysis of MBD IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	6	20.0	--
2	Test Diet	100	30	8	26.7	0.3805
3	Test Diet	500	30	4	13.3	0.8505
4	Test Diet	1000	30	11	36.7	0.1258
5	Heat-treated Control Diet	1000	30	4	13.3	--
6	Positive Control Diet	0[a]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight.
[a] Treatment 6 contained a targeted concentration of 37,500 ng boric acid per mg diet dry weight.

Table 160. Summary Analysis of MBD IPD072Aa Protein Bioassay Weight Change Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Day 0		Day 7		P-Value
				Mean ± Standard Deviation (mg)	Range (mg)	Mean ± Standard Deviation (mg)	Range (mg)	
1	Bioassay Control Diet	0	24	9.38 ± 0.873	8.1 - 11.2	12.4 ± 1.17	10.7 - 16.0	--
2	Test Diet	100	22	9.66 ± 1.19	7.0 - 12.5	12.2 ± 1.20	10.0 - 14.1	0.0252 [a]
3	Test Diet	500	26	9.68 ± 0.809	8.3 - 11.3	12.5 ± 1.12	10.3 - 15.1	0.1667
4	Test Diet	1000	19	9.48 ± 0.741	8.0 - 10.8	12.5 ± 1.14	11.0 - 14.8	0.4681
5	Heat-treated Control Diet	1000	26	9.30 ± 1.03	7.4 - 11.7	12.0 ± 1.35	9.7 - 15.2	--
6	Positive Control Diet	0[b]	0	NA	NA	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight. Not applicable (NA); there were no surviving MBD in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

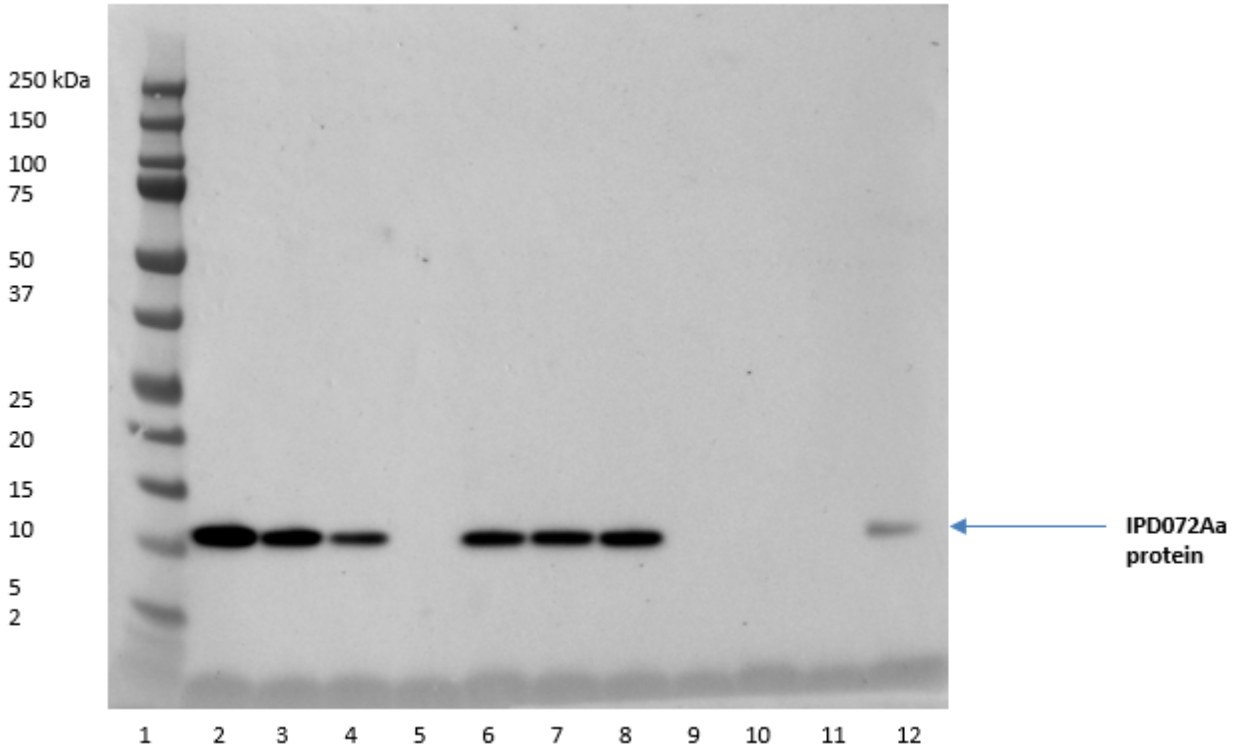
[b] Treatment 6 contained a targeted concentration of 37,500 ng boric acid per mg diet dry weight.

Table 161. Summary of MBD IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0	29[a]	2	6.90
B	Test Diet	85.5	28[a]	28	100

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 4, respectively, of the *Cryptolaemus montrouzieri* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

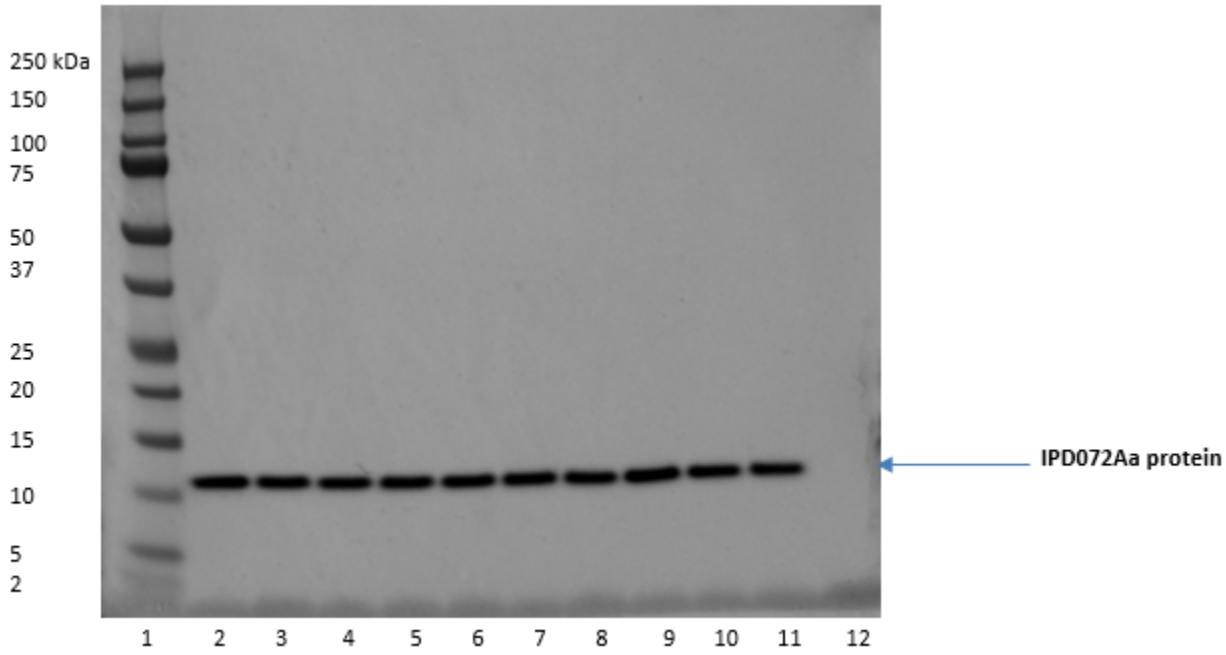
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2)
7	IPD072Aa Protein Test Diet (Treatment 3)
8	IPD072Aa Protein Test Diet (Treatment 4)
9	1X LDS Sample Buffer Blank
10	Bioassay Control Diet (Treatment 1)
11	1X LDS Sample Buffer Blank
12	Heat-Treated Control Diet (Treatment 5)

Note: Nanogram (ng) and lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatments 2, 3, and 4 were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

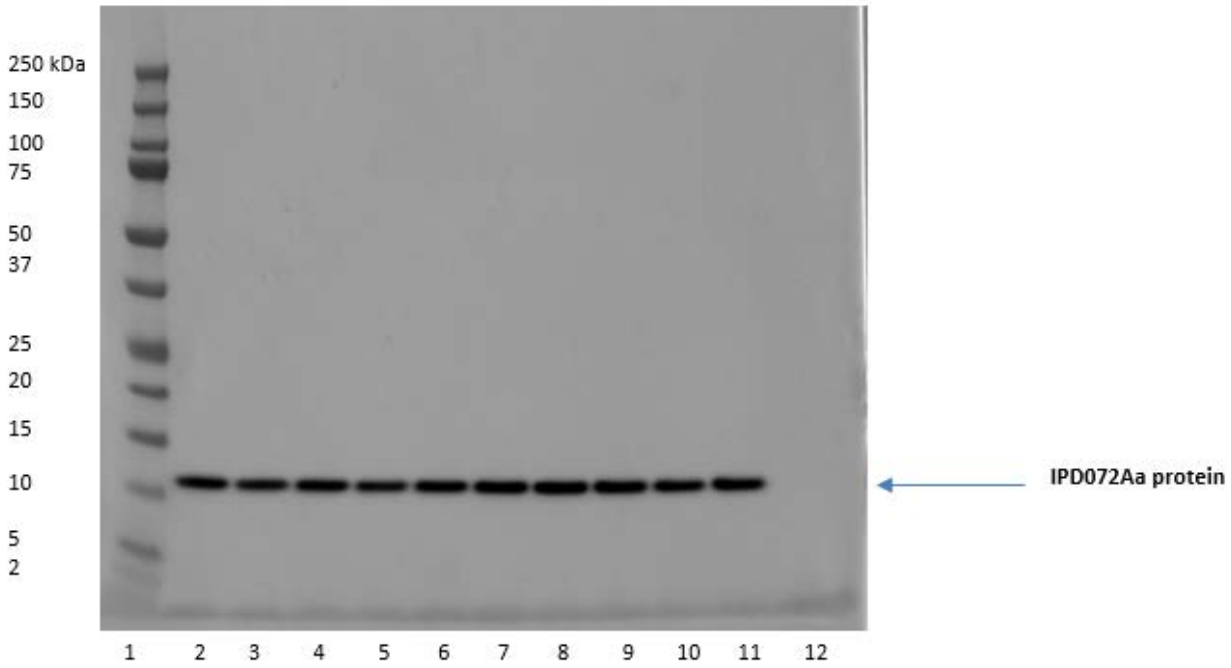
Figure 50. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3, and 4 and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng) and lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

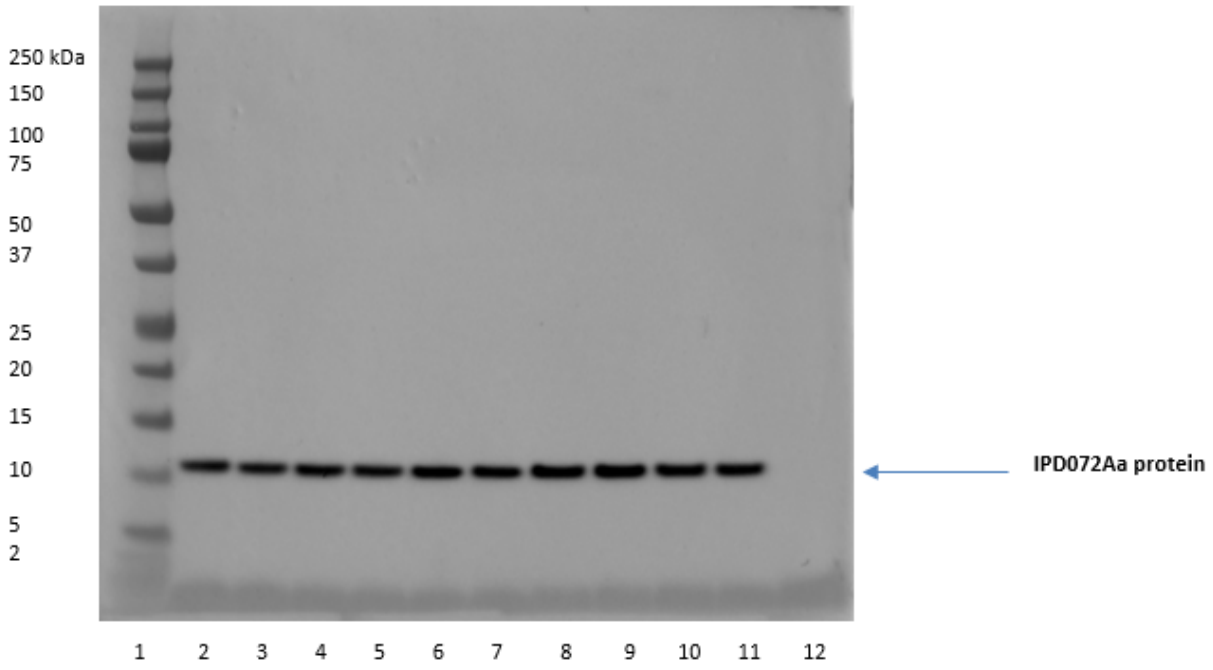
Figure 51. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; End)
10	IPD072Aa Protein Test Diet (Treatment 3; End)
11	IPD072Aa Protein Test Diet (Treatment 3; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng) and lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 3. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

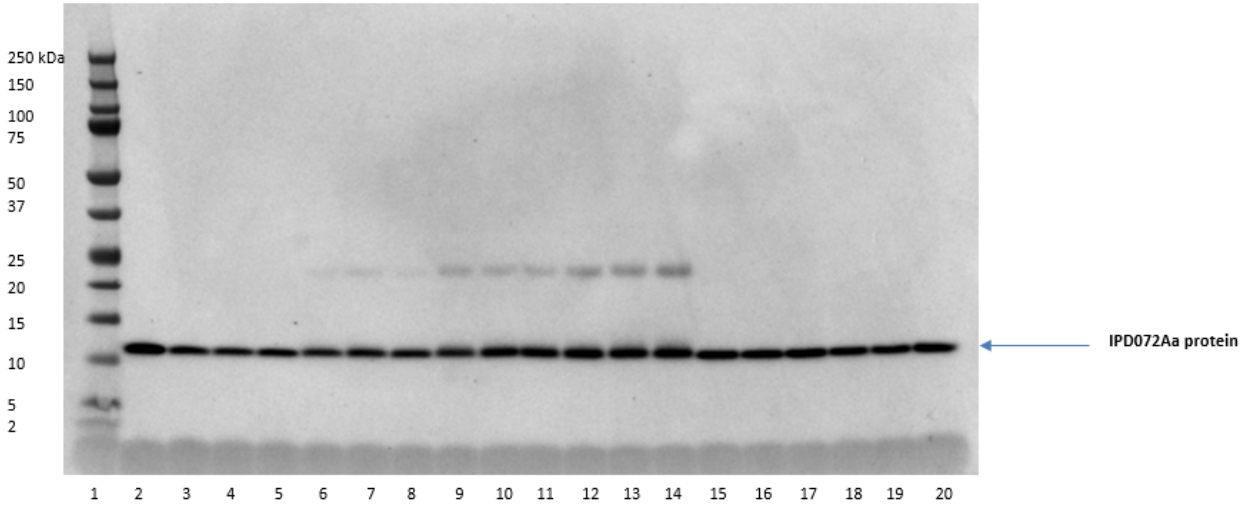
Figure 52. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng) and lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

Figure 53. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	IPD072Aa Protein Test Diet (Bioassay stability; Day 2)
2	Test Substance (10 ng)	12	IPD072Aa Protein Test Diet (Bioassay stability; Day 3)
3	IPD072Aa Protein Test Diet (Bioassay stability; Day 0)	13	IPD072Aa Protein Test Diet (Bioassay stability; Day 3)
4	IPD072Aa Protein Test Diet (Bioassay stability; Day 0)	14	IPD072Aa Protein Test Diet (Bioassay stability; Day 3)
5	IPD072Aa Protein Test Diet (Bioassay stability; Day 0)	15	IPD072Aa Protein Test Diet (Frozen storage stability; Day 0)
6	IPD072Aa Protein Test Diet (Bioassay stability; Day 1)	16	IPD072Aa Protein Test Diet (Frozen storage stability; Day 0)
7	IPD072Aa Protein Test Diet (Bioassay stability; Day 1)	17	IPD072Aa Protein Test Diet (Frozen storage stability; Week 1)
8	IPD072Aa Protein Test Diet (Bioassay stability; Day 1)	18	IPD072Aa Protein Test Diet (Frozen storage stability; Week 1)
9	IPD072Aa Protein Test Diet (Bioassay stability; Day 2)	19	IPD072Aa Protein Test Diet (Frozen storage stability; Week 2)
10	IPD072Aa Protein Test Diet (Bioassay stability; Day 2)	20	IPD072Aa Protein Test Diet (Frozen storage stability; Week 2)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein Treatment 2. Days 0-3 refer to the number of days for which samples were stored under bioassay conditions prior to analysis. Week 1 and Week 2 refer to the approximate length of time samples were stored frozen (-80°C freezer unit) prior to analysis.

Figure 54. Stability Assessment of the IPD072Aa Protein in Treatment 2

F10.a. MBD IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the MBD bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatment 2, 3, and 4 (100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet dry weight, respectively) and the stability under bioassay conditions and the frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively) was also assessed.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation for the MBD bioassay. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.133 mg/ml, 0.667 mg/ml, and 1.33 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the *MBD* bioassay, samples were collected as shown in **Table 156**

Table 162. MBD IPD072Aa Protein Bioassay Diet Samples

Treatment	Number of Samples	Analysis
2	5 beginning	Homogeneity of IPD072Aa protein
	5 middle	
	5 end	
3	5 beginning	
	5 middle	
	5 end	
4	5 beginning	
	5 middle	
	5 end	
2	NA[a]	Visual confirmation of IPD072Aa protein dose
3		
4		
1	1 from each treatment	Verify presence or absence of immunodetectable IPD072Aa protein
5		
1	5 (for Day 0 assessment)	Oven dry weight equivalence
	15 (distributed to dishes)	
2	3 (for Day 0 assessment; homogeneity samples used)	Stability under bioassay conditions
	15 (distributed to dishes)	
2	2 (for Day 0 assessment; homogeneity samples used)	Frozen storage stability (-80 °C freezer unit)
	2 for each timepoint (Week 1 and 2)	

[a] NA (not applicable; a homogeneity sample for each treatment was used for dose confirmation).

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, the aliquots of Treatment 1 and Treatment 2 were removed from storage, distributed to dishes (15 for each treatment) and placed under bioassay conditions using the same methods and conditions used in the *MBD* bioassay. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Days 1-3), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-3) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1, 2, 3, 4, and 5 were weighed to approximately 10 mg on dry ice in preparation for SDS-PAGE. For dose confirmation and assessment of homogeneity in Treatments 2, 3 and 4, Day 0 stability under bioassay conditions and frozen storage stability in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X lithium dodecyl sulfate (LDS) sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing dithiothreitol, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-3), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown in Table 157.

Table 163. MBD IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment	Number of Samples
Dose confirmation of IPD072Aa protein	2	1
	3	1
	4	1
Homogeneity	2	3 beginning
		3 middle
		3 end
	3	3 beginning
		3 middle
		3 end
	4	3 beginning
		3 middle
		3 end
Stability under bioassay conditions	2	3 per timepoint (Days 0-3)
Frozen storage stability	2	2 per timepoint (Day 0, Weeks 1 and 2)
Presence/absence of IPD072Aa protein	1	1
	5	1

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed with

PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:20,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:20,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F10.b. MBD IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the MBD bioassay was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after completion of the MBD bioassay.

The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)

Treatment B: Test Diet (containing a portion of Treatment 4 and targeting 85.5 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 4 during diet aliquoting for the MBD bioassay were removed from the freezer and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the MBD bioassay, resulting in a 30% incorporation of the MBD diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 4 from the MBD bioassay, resulting in a 30% incorporation of the MBD diet by dry weight of the WCR diet.

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 155.

F11. Evaluation of the Survival of Rove Beetle Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the RVB bioassay consisted of Ephestia (Ephestia kuehniella sp.) eggsThe carrier for the sensitive insect WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section F11.a. RVB IPD072Aa Protein Bioassay Analytical Phase

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was *Dalotia coriaria* (rove beetle; Coleoptera: Staphylinidae; RVB). RVB was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. RVB adults were obtained from Rincon-Vitova Insectaries (Ventura, CA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was WCR. The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

RVB adults were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 37,500 ng boric acid per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of six blocks. Each block consisted of a tray containing 1-oz plastic cups and contained five replicates from each treatment. Each treatment was fed to a target of 30 RVB individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photometric cycle of 16 hours of light followed by 8 hours of dark. Adults were refed daily. After 7 days, the bioassay was complete and mortality was assessed.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the RVB bioassay.

Bias in the RVB bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-5 were prepared and maintained as described in the Analytical Phase section below. The boric acid dosing solution for Treatment 6 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 37,500 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 0.75:1 ratio (i.e., 0.75 ml dosing solution to 1 g carrier) to generate Treatments 1-6. After mixing, diets were transferred to 50-ml tubes, lyophilized, and then pooled together by treatment. The bulk diets were aliquoted into individual storage tubes and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1-5 were collected for characterization of diets as described in section F11.a. RVB IPD072Aa Protein Bioassay Analytical Phase. The positive control diet (Treatment 6) was not characterized. A portion each of Treatments 1 and 4 was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay as described in section F11.b. **RVB IPD072Aa Protein** Sensitive Insect Bioassay.

RVB Bioassay

A bioassay was conducted to determine the response of RVB to IPD072Aa protein exposure via oral ingestion. RVB adults were incubated with a water source but no food for at least 24 hours prior to initiation of the bioassay.

On Day 0, diet aliquots were removed from storage and trays were prepared by placing plastic cups in each tray. A moisture source (0.5% agar solution) was added to each plastic cup utilized in the bioassay. Individual Post-it® flags were coated with diet from a single treatment. The diet-coated flags were cut into a maximum of 10 rectangular aliquots of approximately equal size. A rectangular aliquot containing the appropriate diet treatment was distributed to each plastic cup containing agar. One RVB adult was placed in each cup containing diet, and then the cup was sealed with a lid. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photometric cycle of 16 hours of light followed by 8 hours of dark for a total of 7 days. Trays were removed from the environmental chamber and missing or dead organisms were recorded daily. Post-it® flags were replaced with new aliquots as

described for Day 0 and trays were returned to the environmental chamber. One organism in Treatment 1 was not re-fed during the Day 1 refeed but was re-fed on subsequent days. After 7 days, the bioassay was complete and mortality was assessed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variable of interest was mortality. Statistical comparisons were made between *RVB* fed diet containing IPD072Aa protein (Treatments 2, 3, and 4) and the bioassay control diet (Treatment 1).

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

RVB fed an artificial insect diet containing IPD072Aa protein (m_T) (Treatments 2, 3, and 4) was greater than the mortality rate of those fed the bioassay control diet (m_C) (Treatment 1). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Demonstration of IPD072Aa Protein Activity in an RVB Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the RVB bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section below.

Results and Discussion

The RVB bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 6) group exceeded 80%, as shown in Table 164.

The mortality of RVB fed the test diets containing 100 ng/mg, 500 ng/mg, and 1000 ng/mg IPD072Aa protein (Treatments 2, 3, and 4, respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1), as shown in Table 164. Mortality was 3.33% in

Treatment 2 (1 dead; P-value 0.5000), 10.0% in Treatment 3 (3 dead; P-value 0.1186), and 6.67% in Treatment 4 (2 dead; P-value 0.2458). Mortality was 0% in Treatment 1.

Western blot analysis visually confirmed the homogeneity (Figure 55, Figure 56, and Figure 57) and the dose (Figure 58) of the IPD072Aa protein in Treatments 2, 3, and 4. The intensity of the band in Treatment 2 (Figure 58) appeared less intense than Treatments 3 and 4 based on visual analysis. In addition, the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 (Figure 58) was visually confirmed. The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 58); however, the band was less intense than that of the test diets (Treatments 2, 3, and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 58).

Observed larval mortality and weight for the WCR sensitive insect bioassay are summarized in Table 2. The WCR bioassay met the acceptability criterion (F11.b. RVB IPD072Aa Protein Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in Treatment 4 of the RVB bioassay was demonstrated by increased mortality and decreased weight in WCR fed the test diet (Treatment B; described in section F11.b. RVB IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated that exposure to a concentration of 100, 500, or 1000 ng IPD072Aa protein per mg diet (Treatments 2, 3, and 4, respectively) had no adverse effect on the survival of RVB.

Table 164. Summary Analysis of RVB IPD072Aa Protein Bioassay Mortality Results

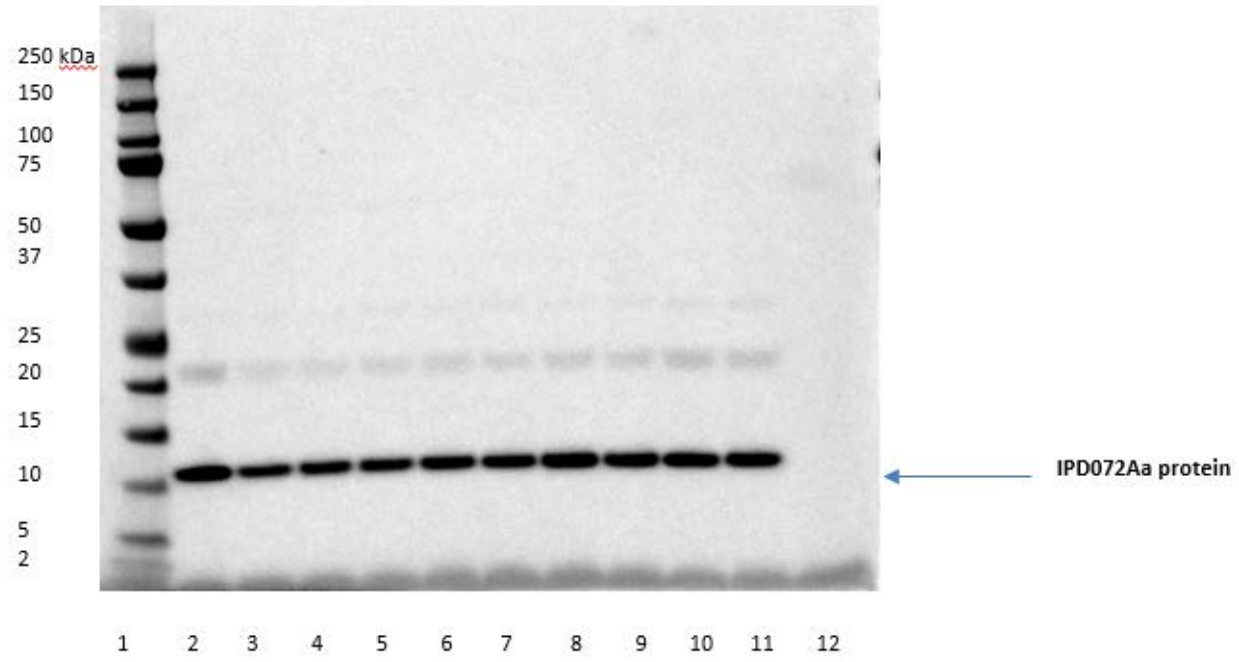
Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	1	3.33	0.5000
3	Test Diet	500	30	3	10.0	0.1186
4	Test Diet	1000	30	2	6.67	0.2458
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0	30	29	96.7	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight. Treatment 6 contained a targeted concentration of 37,500 ng boric acid per mg diet dry weight.

Table 165. Summary of RVB IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	30	3.33	29	0.441 ± 0.159	0.1 - 0.9
B	Test Diet	85.5	30	93.3	2	0.100 ± 0.00	0.1 - 0.1

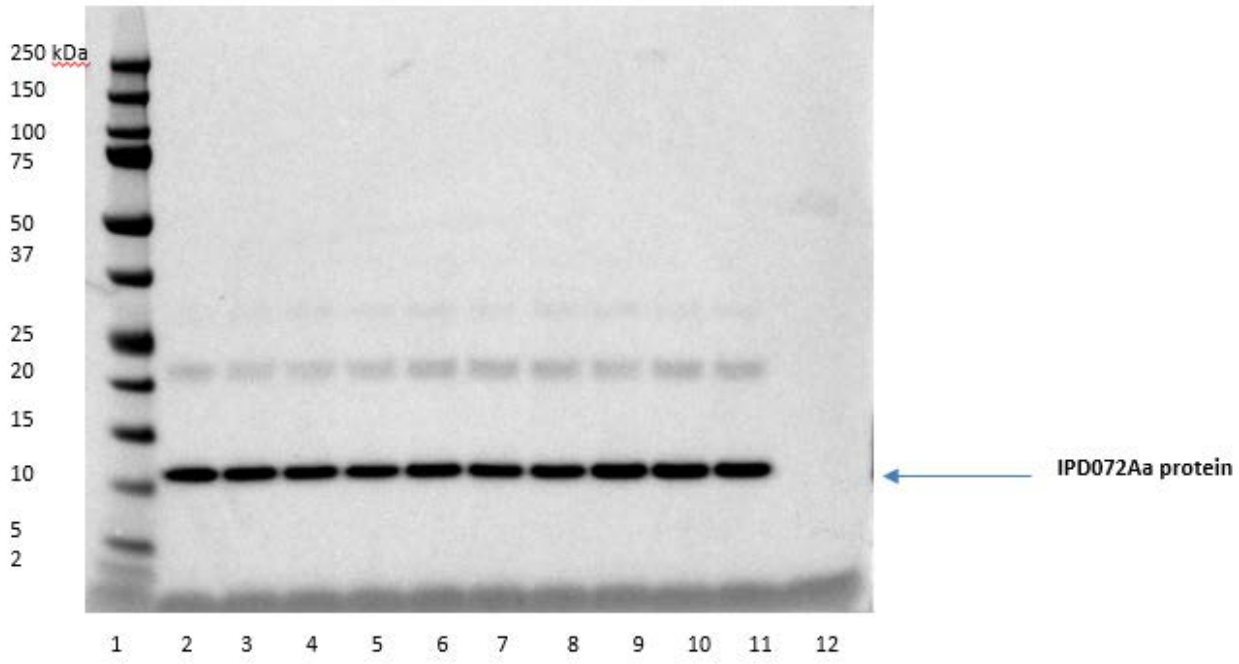
Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera)* bioassay were prepared from the same stored diet preparations used in Treatments 1 and 4, respectively, of the *RVB* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

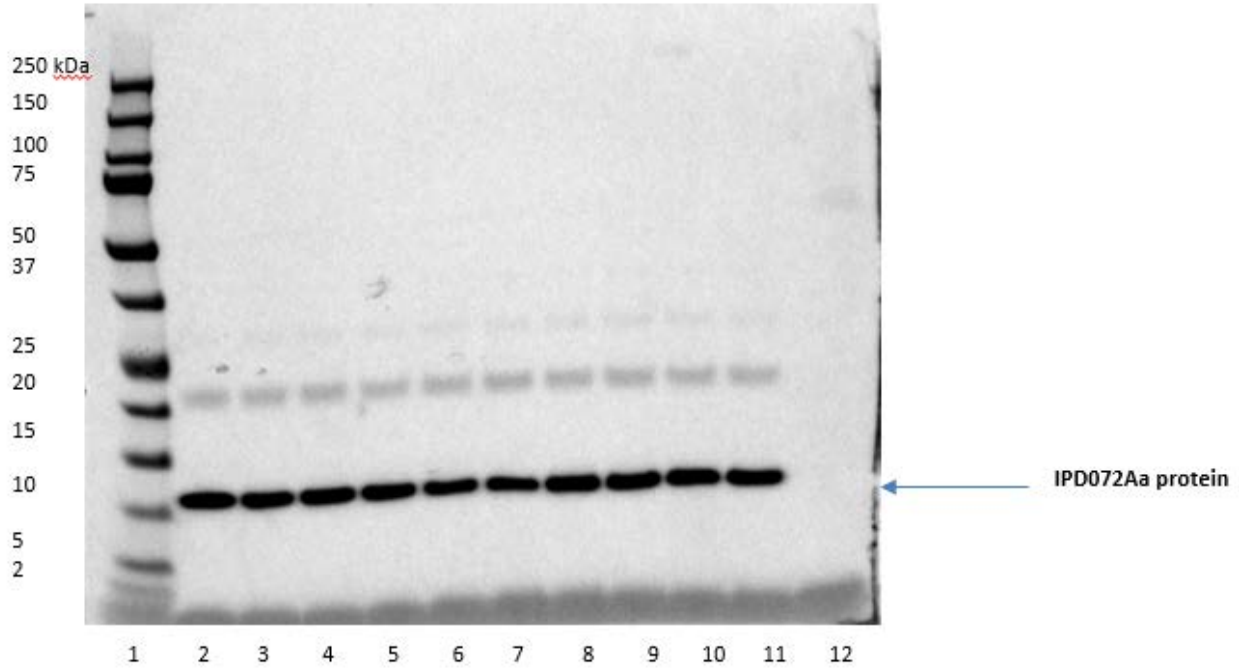
Figure 55. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; End)
10	IPD072Aa Protein Test Diet (Treatment 3; End)
11	IPD072Aa Protein Test Diet (Treatment 3; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 3. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

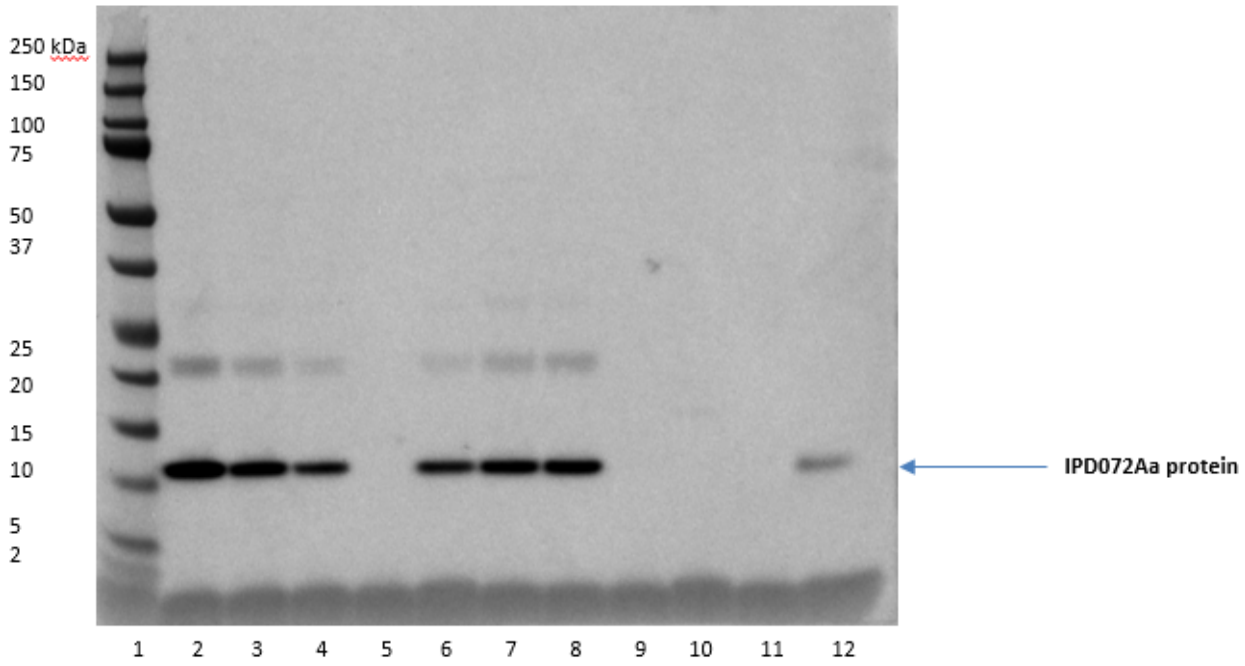
Figure 56. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4. Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

Figure 57. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2)
7	IPD072Aa Protein Test Diet (Treatment 3)
8	IPD072Aa Protein Test Diet (Treatment 4)
9	1X LDS Sample Buffer Blank
10	Bioassay Control Diet (Treatment 1)
11	1X LDS Sample Buffer Blank
12	Heat-Treated Control Diet (Treatment 5)

Note: Nanogram (ng) lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatments 2, 3, and 4 were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

Figure 58. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3 and 4, and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5

F11.a. RVB IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the RVB bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 (100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet dry weight, respectively) and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation. To generate the test dosing solutions for Treatments, 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.133 mg/ml, 0.667 mg/ml, and 1.33 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the RVB bioassay, samples were collected as shown in **Table 166**

Table 166. RVB IPD072Aa Protein Bioassay Diet Samples

Treatment(s)	Number of Samples	Analysis
2, 3, 4	5 beginning (per treatment)	Homogeneity of IPD072Aa protein
	5 middle (per treatment)	
	5 end (per treatment)	
2, 3, 4	NA (Homogeneity sample for each treatment was used)	Visual confirmation of IPD072Aa protein dose
1	1 per treatment	Verify presence or absence of immunodetectable IPD072Aa protein
5		

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1-5 were weighed to approximately 10 mg on wet ice in preparation for SDS-PAGE. Sub-samples were extracted in 600 µl of 1X lithium dodecyl sulfate (LDS) sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing dithiothreitol, and 65% water). In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, and thawed samples were re-heated at 90-100 °C for 3 minutes. The following samples were then loaded into 4-12% Bis-Tris gelsas shown in Table 167.

Table 167. RVB IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment(s)	Number of Samples
Homogeneity	2, 3, 4	3 beginning (per treatment)
		3 middle (per treatment)
		3 end (per treatment)
Dose confirmation of IPD072Aa protein	2, 3, 4	1 per treatment
Presence/absence of IPD072Aa protein	1	1
	5	1

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel

electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for 50 minutes at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 50 minutes at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

F11.b. RVB IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the RVB bioassay was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The *WCR* bioassay was initiated after completion of the RVB bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 30% Treatment 1 from the RVB bioassay by dry weight of WCR diet)

Treatment B: Test Diet (containing 30% Treatment 4 from the RVB bioassay by dry weight of WCR diet and targeting 85.5 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 4 during diet preparation for the RVB bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the RVB bioassay, resulting in a 30% incorporation of the RVB diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 4 from the RVB bioassay, resulting in a 30% incorporation of the RVB diet by dry weight of the WCR diet.

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were

placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 2. Weight data were summarized as means, standard deviations, and ranges and are presented in Table 165.

F12. Evaluation of the Survival and Weight of European Corn Borer Fed an Artificial Diet Containing Purified IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unitstorage condition.

Carrier

The carrier for the ECB bioassay consisted of Stonefly Heliothis diet.

The carrier for the WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solution

The bioassay control dosing solution consisted of ultrapure water.

The test dosing solution consisted of the test substance diluted in ultrapure water to achieve the concentration in the test diet.

The heat-treated control dosing solution consisted of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes as detailed in t section F12.a. ECB IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was European corn borer (Ostrinia nubilalis, Lepidoptera: Crambidae; ECB). ECB was selected as a representative lepidopteran to characterize the spectrum of activity of IPD072Aa protein. ECB eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was western corn rootworm (Diabrotica virgifera virgifera, Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

ECB larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet targeting 1000 ng IPD072Aa protein per mg diet wet weight
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (targeting 3750 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained three replicates from each treatment. Each treatment was fed to a target of 30 ECB individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 4 and missing and dead organisms were recorded. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between ECB provided Treatments 1 and 2.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.

The mortality of the positive control diet (Treatment 4) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diet. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets were also assessed. Stability of IPD072Aa protein in the carrier under bioassay conditions was confirmed under a separate study and was not reassessed during this study. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in the ECB bioassay test diet.

Bias in the ECB bioassay and sensitive insect bioassay portions of this study was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing and appropriate assay controls.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

On each day of diet preparation, dosing solutions for Treatments 1, 2, and 3 were prepared as described in section F12.a. ECB IPD072Aa Protein Bioassay Analytical Phase. The boric acid dosing solution for Treatment 4 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a target concentration of 3750 ng/mg diet wet weight. Each dosing solution was mixed with carrier in a 3:1 ratio (i.e., 3 ml dosing solution to 1 g carrier) to generate Treatments 1-4.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1, 2, and 3 were collected for characterization of diets as described in section F12.b. ECB IPD072Aa Protein Sensitive Insect BioassayThe positive control diet (Treatment 4) was not characterized. A portion of Treatment 1 and 2 from each day of diet preparation was collected for use in diets for the sensitive insect bioassay as described in the section F12.b. ECB IPD072Aa Protein Sensitive Insect Bioassay.

ECB Bioassay

A bioassay was conducted to determine the response of ECB to IPD072Aa protein exposure via oral ingestion. ECB eggs were incubated in an environmental chamber until the eggs hatched. ECB neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One ECB neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 7 days. On Day 4, new bioassay plates were prepared as described for Day 0, living ECB larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparison was made between ECB fed diet containing IPD072Aa protein (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of *ECB* fed the artificial insect diet containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2; therefore, a two-sample *t*-test was conducted to test if exposure to IPD072Aa protein caused growth inhibition. That is, to test if the weight of *ECB* fed the artificial insect diet containing IPD072Aa protein (w_T) was less than the weight of those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across treatment groups 1 and 2 was used. A significant difference was established if the P-value was < 0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Demonstration of IPD072Aa Protein Activity in the *ECB* Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 2 in the ECB bioassay. The initial sensitive insect bioassay did not meet the acceptability criteria; therefore, the entire study was re-run. Details regarding the second sensitive insect bioassay are provided in section F12.b. ECB IPD072Aa Protein Sensitive Insect Bioassay.

Results and Discussion

The ECB bioassay met the acceptability criteria as the combined dead and missing count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 168.

However, the ECB bioassay was run twice. On the initial run, the sensitive insect bioassay used to demonstrate the biological activity of the IPD072Aa protein in the test diet used in the ECB bioassay did not meet the acceptability criterion. The ECB bioassay was re-run using freshly prepared dosing solutions and diets and the sensitive insect bioassay was repeated. The second run of the sensitive insect bioassay met the acceptability criterion; therefore, results for the second run of the ECB bioassay are included in this report. Data generated for the initial runs of the ECB and WCR bioassays, as well as diet characterization results for the first ECB bioassay, were not included in this report but were retained with the study records.

The mortality of ECB fed the test diet containing IPD072Aa protein (Treatment 2; 0 dead; 0% mortality) was not significantly greater than those fed the bioassay control diet (Treatment 1; 2 dead; 6.67% mortality; Fisher's exact test P-value = 1.0000), as shown in Table 168.

The mean weight of ECB fed the test diet containing IPD072Aa protein (Treatment 2; 6.72 mg) was not significantly less than those fed the bioassay control diet (Treatment 1; 6.70 mg; t-Test P-value = 0.5102), as shown in Table 169.

Western blot analysis visually confirmed the dose and homogeneity of the IPD072Aa protein in the test diet (Treatment 2; Figure 59, Figure 60, and Figure 61). Homogeneity assessment on one day of diet preparation is considered representative of both days of diet preparation. The IPD072Aa protein was detected in the heat-treated control diet (Treatment 3; Figure 59; Figure 60), but the band was less intense than that of the test diet (Treatment 2). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 59; Figure 60)

Observed larval mortality and weight data for the second run of the WCR sensitive insect bioassay are summarized in Table 170. The biological activity of the IPD072Aa protein in the

test diet used in the ECB bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in section F12.b. ECB IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on survival or weight of ECB.

Table 168. Summary Analysis of ECB IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	30	2	6.67	1.0000
2	Test Diet	30	0	0	
3	Heat-treated Control Diet	30	0	0	--
4	Positive Control Diet	30	30	100	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

Table 169. Summary Analysis of ECB IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	*t*-Test P-Value
1	Bioassay Control Diet	28	6.70 (5.47 - 7.92)	0 - 12.7	0.5102
2	Test Diet	30	6.72 (5.64 - 7.79)	0.3 - 10.9	
3	Heat-Treated Control Diet	30	6.46 ± 2.34[a]	2.1 - 9.5	--
4	Positive Control Diet	0	NA	NA	--

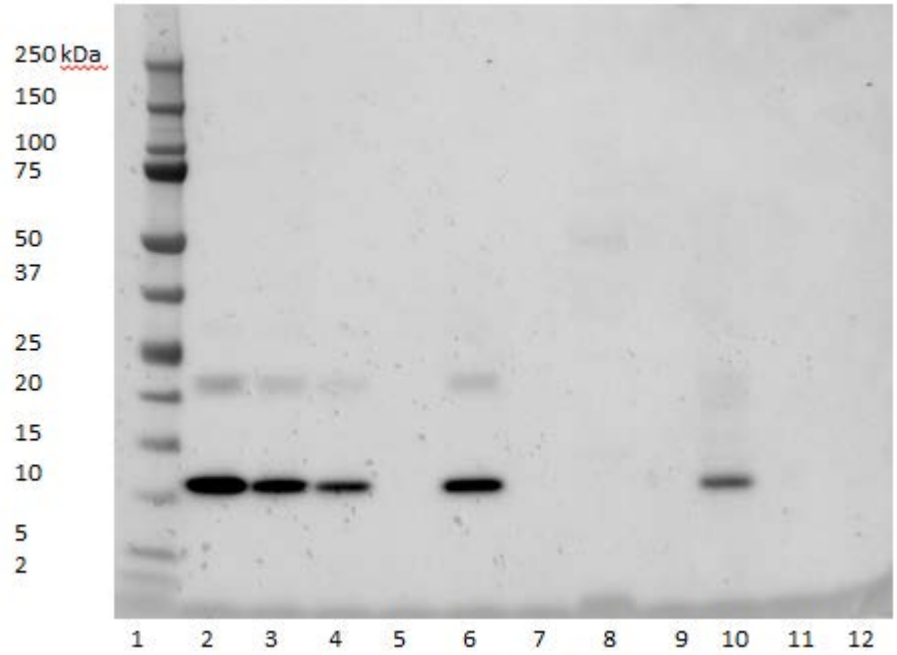
Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight. Not applicable (NA); there were no surviving ECB in the positive control diet group.
[a] Standard deviation is provided for mean values not subjected to a *t*-test.

Table 170. Summary of ECB IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0 ng IPD072Aa/mg	30	0.00	30	0.520 ± 0.177	0.100 - 0.900
B	Test Diet	100 ng IPD072Aa/mg	29[a]	93.1	2	0.150 ± 0.0707	0.100 - 0.200

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the *Ostrinia nubilalis* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the corn rootworm artificial diet.

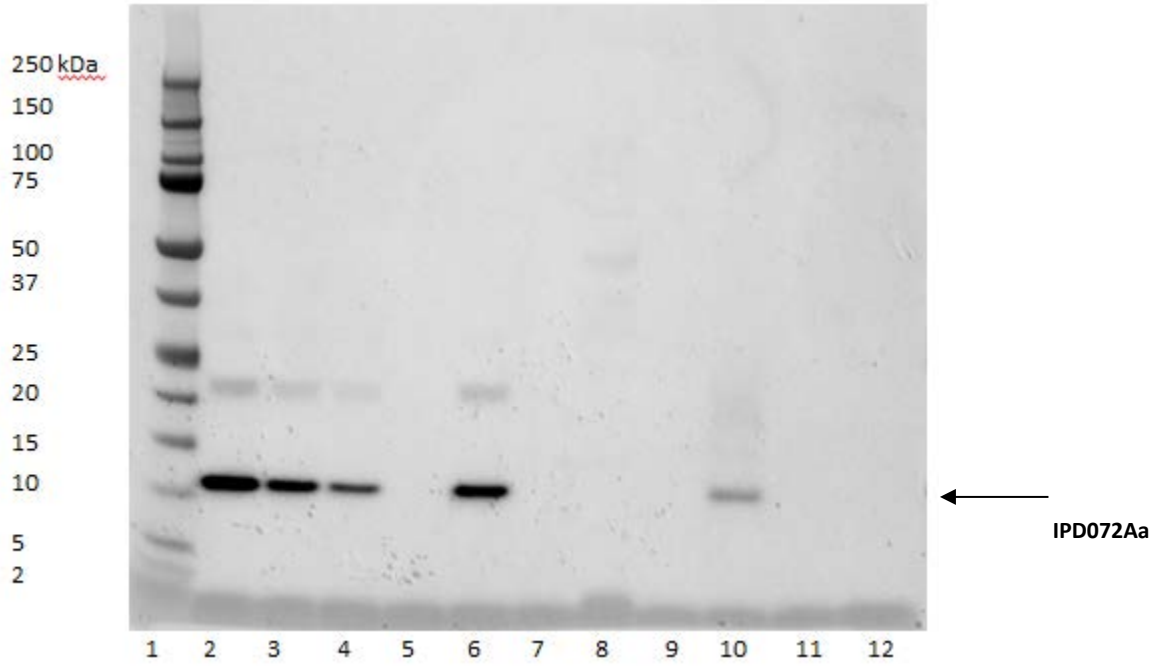
[a] Organisms counted as missing during the bioassay, or wells that contained more than one organism, were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-Stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
7	1X LDS Sample Buffer Blank
8	Bioassay Control Diet (Treatment 1; undiluted)
9	1X LDS Sample Buffer Blank)
10	Heat-Treated Control Diet (Treatment 3; 10 ng)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lane 8 are attributed to non-specific binding to diet components in the more concentrated bioassay control diet analytical sample (~130x relative to Lanes 6 and 10).

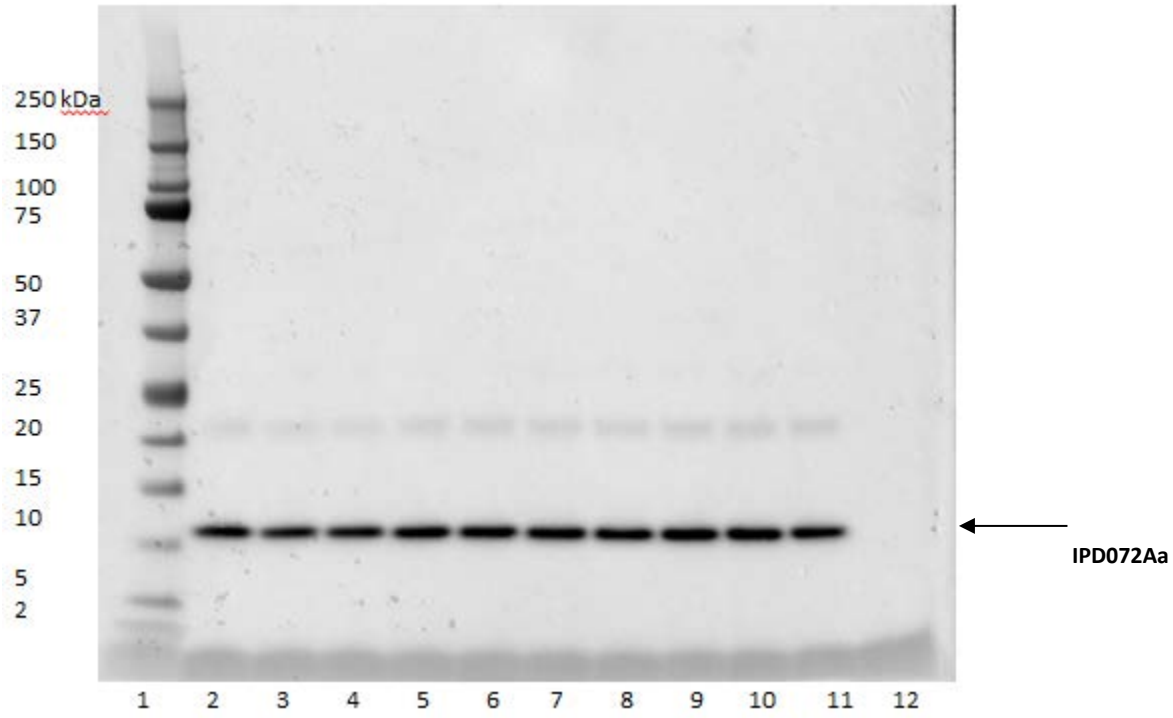
Figure 59. Dose Confirmation of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in the Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3), Day 0



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
7	1X LDS Sample Buffer Blank
8	Bioassay Control Diet (Treatment 1; undiluted)
9	1X LDS Sample Buffer Blank)
10	Heat-Treated Control Diet (Treatment 3; 10 ng)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lane 8 are attributed to non-specific binding to diet components in the more concentrated bioassay control diet analytical sample (~130x relative to Lanes 6 and 10).

Figure 60. Dose Confirmation of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in the Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3), Day 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

Figure 61. Homogeneity Assessment of the IPD072Aa Protein in the Test Diet (Treatment 2)

F12.a. ECB IPD072Aa Protein Bioassay Analytical Phase

The following dosing solutions were prepared for the ECB bioassay:

Bioassay control dosing solution consisting of chilled ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solution consisting of IPD072Aa protein test substance diluted in ultrapure water

Heat-treated IPD072Aa protein control dosing solution consisting of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose (1000 ng IPD072Aa protein per mg diet wet weight) and homogeneity of the IPD072Aa protein in the test diet (Treatment 2 in the ECB bioassay). Homogeneity assessment on one day of diet preparation is considered representative of both days of diet preparation. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 3, respectively, in the ECB bioassay) were also assessed.

Preparation of Solutions

Dosing solutions were prepared on Day 0 and Day 4 of the ECB bioassay and maintained chilled until use. To generate the test dosing solution, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to an IPD072Aa protein concentration of 1.33 mg/ml. To generate the heat-treated IPD072Aa protein dosing solution, a portion of the test dosing solution for each of Day 0 and Day 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution for the ECB bioassay, samples of Treatments 1, 2, and 3 were collected as follows:

On Day 0, 15 samples of Treatment 2 were collected, five each from the beginning, middle, and end of the diet preparation process, to assess homogeneity of the IPD072Aa protein in the test

diet. One of the samples collected for homogeneity was also used for visual confirmation of the dose of IPD072Aa protein in the Day 0 test diet.

On Day 0 and Day 4, one sample each of Treatment 1 and Treatment 3 were collected to verify the presence or absence of immunodetectable IPD072Aa protein.

On Day 4, one sample of Treatment 2 was collected for visual confirmation of the dose of IPD072Aa protein in the Day 4 test diet.

Sample Extraction and SDS-PAGE

Sub-samples of Treatments 1, 2, and 3 were weighed to approximately 40 mg each on wet ice in preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as follows:

For visual confirmation of the dose of IPD072Aa protein in the test diet and assessment of the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3, dilutions of the test substance were loaded (5, 10, and 20 ng) to gels (one gel for Day 0 samples and one for Day 4 samples). Treatment 2 and Treatment 3 (10 ng each based on nominal concentration) were also loaded to each gel. Treatment 1 was loaded to each gel undiluted.

For homogeneity assessment, nine samples of Treatment 2 (three each from the beginning, middle, and end of the Day 0 diet distribution process) and a sample of the test substance were loaded to a gel at 10 ng each.

Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Protein aggregation such as dimer formation may be observed; an IPD072Aa protein dimer would be expected to migrate at approximately 20 kDa.

F12.b. ECB IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 2 used in the Ostrinia nubilalis (ECB) bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera virgifera (WCR, Coleoptera: Chrysomelidae), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on Day 0 of the Ostrinia nubilalis bioassay.

WCR neonates were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 10% Treatment 1 from the ECB bioassay by wet weight of WCR diet)

Treatment B: Test Diet (containing 10% Treatment 2 from the ECB bioassay by wet weight of diet; targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Day 0 and Day 4 diets prepared for the WCR bioassay contained a portion of Day 0 and Day 4 diets, respectively, prepared for the ECB bioassay. Collected samples of Treatments 1 and 2 from each day of diet preparation for the ECB bioassay were used to prepare Treatments A and B as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with corn rootworm artificial diet at a 2.5:1 ratio (i.e., 2.5 ml water to 1 g carrier).

For Treatment A, the wet corn rootworm diet was mixed with Treatment 1 from the ECB bioassay, resulting in a 10% incorporation of the ECB diet by wet weight of the WCR diet.

For Treatment B, the wet corn rootworm diet was mixed with Treatment 2 from the ECB bioassay, resulting in a 10% incorporation of the ECB diet by wet weight of the WCR diet.

WCR

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed,

and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from reporting.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 170. Weight data were summarized as means, standard deviations, and ranges and are presented in Table 170.

F13. Evaluation of the Survival and Weight of Corn Earworm Fed an Artificial Diet Containing Purified IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

An artificial insect diet consisting of Stonefly Heliothis diet was used as the carrier into which the dosing solutions were incorporated for the CEW bioassay.

The carrier for the sensitive insect bioassay was a corn rootworm artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution consisted of ultrapure water.

The test dosing solution consisted of the test substance diluted in ultrapure water to achieve the concentration in the test diet.

The heat-treated control dosing solution consisted of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes as described in section F13.a. CEW IPD072Aa Bioassay Analytical Phase.

The positive control dosing solution consisted of boric acid (H_3BO_3; Sigma-Aldrich) and ultrapure water.

Test System

The test system was corn earworm (Helicoverpa zea; Lepidoptera: Noctuidae; CEW). CEW was selected as a representative lepidopteran to characterize the spectrum of activity of IPD072Aa protein. CEW eggs were obtained from Benzon Research Inc. (Carlisle, PA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect portion of the study was western corn rootworm (Diabrotica virgifera virgifera; Coleoptera: Chrysomelidae). The test system was chosen

because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

CEW larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet targeting 1000 ng IPD072Aa protein per mg diet wet weight
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (containing boric acid)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained three replicates from each treatment. Each treatment was fed to a target of 30 CEW individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle. Larvae were refed on Day 4 and missing and dead organisms were recorded. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between CEW provided Treatments 1 and 2.

A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in the test diet. Western blot analysis was used to visually confirm the dose, homogeneity, and stability under bioassay conditions of the IPD072Aa protein in the test diet. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets was also assessed.

Bias in the CEW bioassay and the sensitive insect bioassay portions of this study was controlled through the randomization of treatments within blocks and the use of control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing and appropriate assay controls.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Dosing solutions for Treatments 1, 2, and 3 were prepared as described in section F13.a. CEW IPD072Aa Bioassay Analytical Phase. The boric acid dosing solution for Treatment 4 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a target concentration of 3125 ng/mg diet wet weight. Each dosing solution was mixed with carrier in a 3:1 ratio (i.e., 3 ml dosing solution to 1 g carrier) to generate Treatments 1-4.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1, 2, and 3 were collected for characterization of diets as described in Appendix A. The positive control diet (Treatment 4) was not characterized. A portion of Treatment 1 and Treatment 2 from each day of diet preparation was collected for use in diets in the sensitive insect bioassay as described in section F13.b. CEW IPD072Aa Bioassay Sensitive Insect Bioassay.

CEW Bioassay

A bioassay was conducted to determine the response of CEW to IPD072Aa protein exposure via oral ingestion. CEW eggs were incubated in an environmental chamber until the eggs hatched. CEW neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One CEW neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 7 days. On Day 4, new bioassay plates were prepared, as described for Day 0, with the exception that 600 µl of freshly prepared diet were dispensed to each well. Living CEW larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well or wells containing more than one organism, were excluded from reporting.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.

The mortality of the positive control diet (Treatment 4) group does not exceed 80%.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.3 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparison was made between CEW fed diet containing IPD072Aa protein (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of CEW fed the artificial insect diet containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of treatment groups 1 and 2; therefore, a two-sample *t*-test was conducted to test if exposure to IPD072Aa protein caused growth inhibition. That is, to test if the weight of CEW fed the artificial insect diet containing IPD072Aa protein (w_T) was less than the weight of those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across treatment groups 1 and 2 was used. A significant difference was established if the

P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Demonstration of IPD072Aa Protein Activity in the CEW Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 2 in the CEW bioassay. Details regarding the sensitive insect bioassay are provided in section F13.b. CEW IPD072Aa Bioassay Sensitive Insect Bioassay.

Results and Discussion

For the CEW bioassay (Table 1), the bioassay control diet (Treatment 1) group met the acceptability criteria as the combined dead and missing CEW count did not exceed 20%. The positive control diet (Treatment 4) group did not meet the acceptability criterion of exceeding 80% mortality; however, compared to Treatment 1, the weights observed in the positive control group were substantially less, indicating clear growth inhibition. The increased mortality and decreased weight of CEW in Treatment 4 as compared with Treatment 1 indicates CEW were exposed to the boric acid in Treatment 4 by feeding on the provided diet, which demonstrates the experimental design was appropriate.

The mortality of CEW fed the test diet containing IPD072Aa protein (Treatment 2; 0 dead; 0% mortality) was not significantly greater than those fed the bioassay control diet (Treatment 1; 0 dead; 0% mortality; Fisher's exact test P-value = 1.0000), as shown in Table 171.

The mean weight of CEW fed the test diet containing IPD072Aa protein (Treatment 2; 97.5 mg) was not significantly different than the mean weight of those fed the bioassay control diet (Treatment 1; 109 mg; t-test P-value= 0.0704), as shown in Table 2.

Western blot analysis visually confirmed the dose (Day 0 and 4), homogeneity, and stability of the IPD072Aa protein in the test diet under bioassay conditions (Treatment 2; Figure 62; Figure 63; Figure 64; Figure 65). Homogeneity assessment on one day of diet preparation is considered representative of both days of diet preparation. The IPD072Aa protein was detected in the heat-treated control diet (Treatment 3, Figure 62; Figure 63), but the band was less intense than that of the test diet (Treatment 2). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 62; Figure 63).

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 3. The WCR bioassay met the acceptability criterion (section F13.b. CEW IPD072Aa Bioassay Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in the test diet used in the CEW bioassay was demonstrated by 100% mortality of WCR fed the test diet (Treatment B; described in the Sensitive Insect Bioassay section below).

Conclusion

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on the survival or weight of CEW.

Table 171. Summary Analysis of CEW IPD072Aa Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	30	0	0	1.0000
2	Test Diet	30	0	0	
3	Heat-treated Control Diet	30	2	6.67	--
4	Positive Control Diet	30	14	46.7	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

Table 172. Summary Analysis of CEW IPD072Aa Bioassay Weight Results

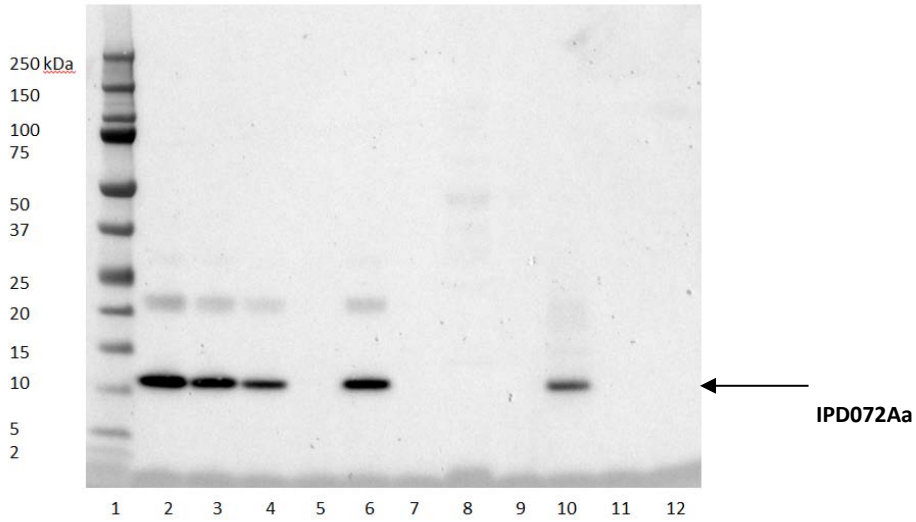
Treatment	Treatment Description	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	*t*-Test P-Value
1	Bioassay Control Diet	30	109 (98.4 - 119)	56.2 - 151.7	0.0704
2	Test Diet	30	97.5 (86.1 - 109)	11.6 - 162.4	
3	Heat-treated Control Diet	28	95.5 ± 42.3[a]	26.1 - 184.5	--
4	Positive Control Diet	16	0.325 ± 0.139[a]	0.1 - 0.6	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.
[a] Standard deviation is provided for mean values not subjected to a *t*-test.

Table 173. Summary of CEW IPD072Aa Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0 ng IPD072Aa/mg	26[a]	2	7.69
B	Test Diet	100 ng IPD072Aa/mg	28[a]	28	100

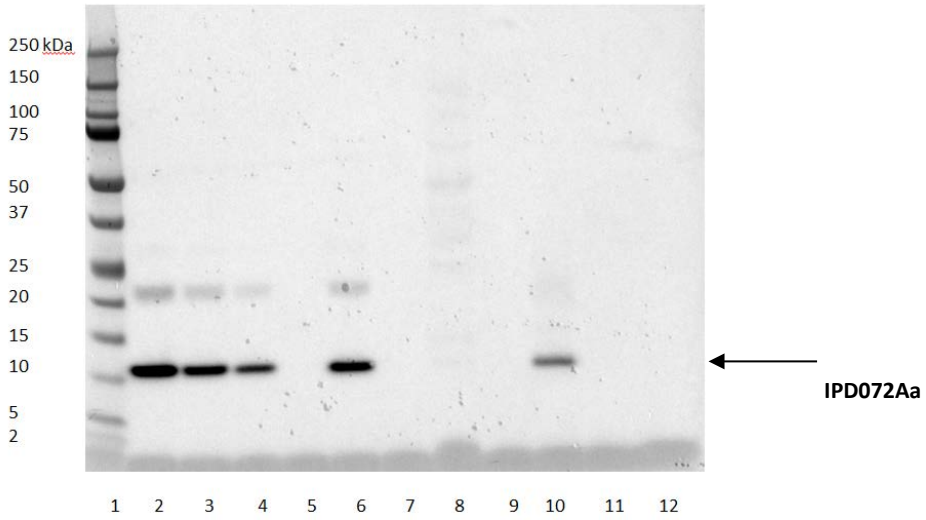
Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared with the same diet preparations used in Treatments 1 and 2, respectively, of the CEW bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the corn rootworm artificial diet.
[a] Organisms counted as missing during the bioassay, or wells that contained more than one organism, were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
7	1X LDS Sample Buffer Blank
8	Bioassay Control Diet (Treatment 1; undiluted)
9	1X LDS Sample Buffer Blank
10	Heat-Treated Control Diet (Treatment 3; 10 ng)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test diet and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lane 8 are attributed to non-specific binding to diet components in the more concentrated control diet analytical sample (~130x relative to Lanes 6 and 10).

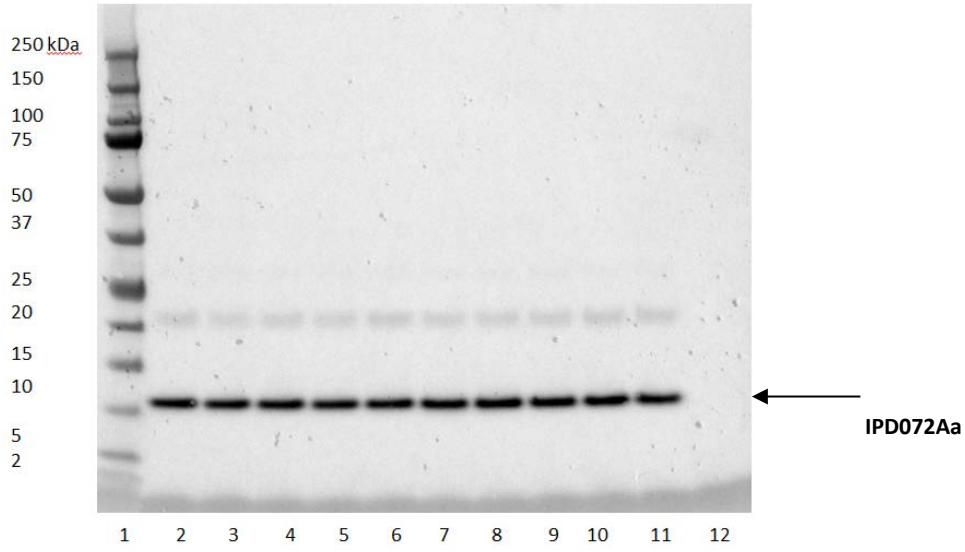
Figure 62. Dose Confirmation of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodectable IPD072Aa Protein in the Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3), Day 0



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
7	1X LDS Sample Buffer Blank
8	Bioassay Control Diet (Treatment 1; undiluted)
9	1X LDS Sample Buffer Blank
10	Heat-Treated Control Diet (Treatment 3; 10 ng)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test diet and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lane 8 are attributed to non-specific binding to diet components in the more concentrated control diet analytical sample (~130x relative to Lanes 6 and 10).

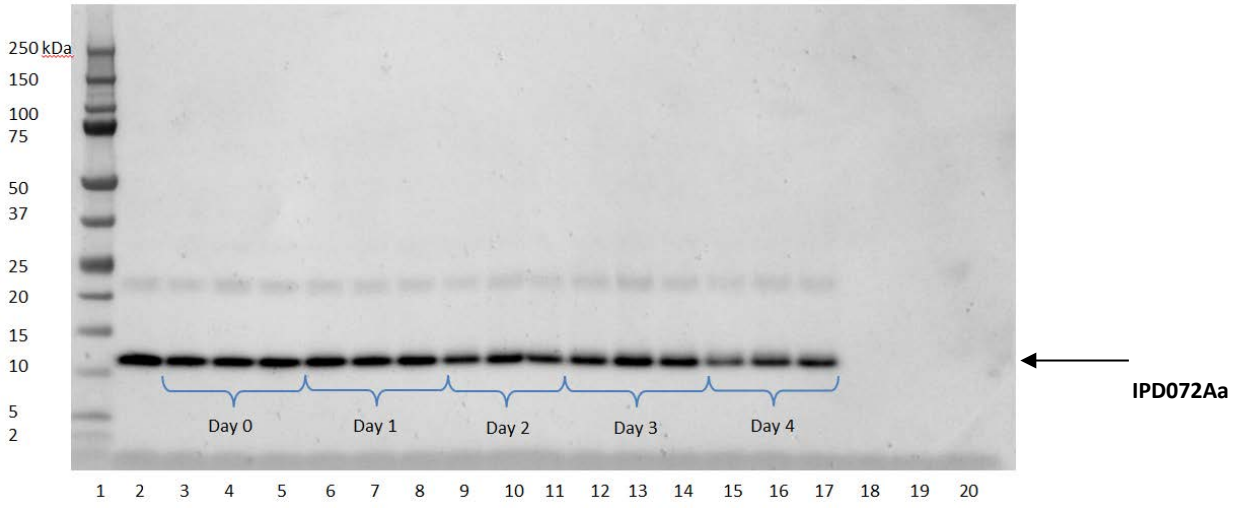
Figure 63. Dose Confirmation of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodectable IPD072Aa Protein in the Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3), Day 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Beginning, middle, and end refer to the stages in the diet aliquoting process at which samples were collected.

Figure 64. Homogeneity Assessment of the IPD072Aa Protein in the Test Diet (Treatment 2)



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
18	1X LDS Sample Buffer Blank
19	1X LDS Sample Buffer Blank
20	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). Days 0-4 refers to the number of days samples were stored under bioassay conditions prior to analysis. On Day 4 of stability analysis, one out of three of the IPD072Aa protein bands appeared slightly less intense than on Days 0-3; however, organisms were refed every 3-4 days.

Figure 65. Stability Assessment of the IPD072Aa Protein in the Test Diet (Treatment 2) under Bioassay Conditions

F13.a. CEW IPD072Aa Bioassay Analytical Phase

The following dosing solutions were prepared for the CEW bioassay:

- Bioassay control dosing solution consisting of chilled ultrapure (American Society for Testing and Materials (ASTM) Type 1) water
- Test dosing solution consisting of IPD072Aa protein test substance diluted in ultrapure water
- Heat-treated IPD072Aa protein control dosing solution consisting of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose (1000 ng IPD072Aa protein per mg diet dry weight), homogeneity, and stability under bioassay conditions of the IPD072Aa protein in the test diet (Treatment 2 in the CEW bioassay). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 3, respectively, in the CEW bioassay) was also assessed.

Preparation of Solutions

Dosing solutions were prepared on Day 0 and Day 4 of the CEW bioassay and maintained chilled until use. To generate the test dosing solutions, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to an IPD072Aa protein concentration of 1.33 mg/ml. To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution for each of Day 0 and Day 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution, samples of Treatments 1, 2, and 3 were collected as follows:

- On Day 0 of the CEW bioassay diet distribution process, 15 samples of Treatment 2 were collected, five each from the beginning, middle, and end of the process to assess homogeneity of IPD072Aa protein in the test diet. Some of the samples were also used for visual confirmation of the dose of IPD072Aa protein in the Day 0 test diet.
- On Day 0 and Day 4 of the CEW bioassay diet distribution process, a sample each of Treatment 1 and Treatment 3 were collected to verify the presence or absence of immunodetectable IPD072Aa protein.
- On Day 4 of the CEW bioassay diet distribution process, three samples each of Treatment 1 and Treatment 2 were collected and used for Day 0 samples for oven dry weight equivalence and bioassay stability, respectively.

One of the Treatment 2 samples was also used for visual confirmation of the dose of IPD072Aa protein in the Day 4 test diet.

- On Day 4 of the CEW bioassay diet distribution process, 20 aliquots each of Treatment 1 and Treatment 2 were distributed to bioassay plates for bioassay stability.

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, samples of Treatment 1 and Treatment 2 (20 each) distributed to bioassay plates were placed under bioassay conditions using the same methods and conditions used in the CEW bioassay. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Day 1, 2, 3, and 4), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. The Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability of Treatment 2 under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of at least three Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and SDS-PAGE

Samples of Treatments 1, 2, and 3 were weighed to approximately 40 mg each on wet ice in preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For dose confirmation and assessment of homogeneity and stability under bioassay conditions (Day 0) in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3, samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2

(Days 1-4), samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each timepoint. In addition, undiluted samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as follows:

- For visual confirmation of the dose of IPD072Aa protein in the test diet and assessment of the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3, dilutions of the test substance were loaded (5, 10, and 20 ng) to gels (one gel for Day 0 samples and one for Day 4 samples). Samples of Treatment 2 and Treatment 3 (10 ng each based on nominal concentration) were also loaded to each gel. Samples of Treatment 1 were loaded to each gel undiluted.

- For homogeneity assessment, nine samples of Treatment 2 (three each from the beginning, middle, and end of the diet distribution process) and a sample of the test substance were loaded to a gel at 10 ng each.

- For assessment of stability under bioassay conditions, Treatment 2 samples were loaded to a gel at 10 ng (based on nominal concentration of Treatment 2) for each sampling time point (Days 0-4; three samples each). A sample of the test substance (10 ng) was also loaded.

Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a Mini-Cell or Midi-Cell Electrophoresis System with 1X MES running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed three times for 1 minute each with PBST to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. The blots remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Protein aggregation such as dimer formation may be observed; an IPD072Aa protein dimer would be expected to migrate at approximately 20 kDa.

F13.b. CEW IPD072Aa Bioassay Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 2 used in the Helicoverpa zea (CEW) bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera virgifera (WCR), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on Day 0 of the CEW bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

- Treatment A: Bioassay Control Diet (containing 10% Treatment 1 from the CEW bioassay by wet weight of WCR diet)
- Treatment B: Test Diet (containing 10% Treatment 2 from the CEW bioassay by wet weight of diet; targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Day 0 and Day 4 diets prepared for the WCR bioassay contained a portion of Day 0 and Day 4 diets, respectively, prepared for the CEW bioassay. Collected samples of Treatments 1 and 2 from each day of diet preparation for the CEW bioassay were used to prepare Treatments A and B as follows:

- For each respective treatment, ultrapure water (American Society for Testing and Materials ASTM Type 1 water) was mixed with corn rootworm artificial diet at a 2.5:1 ratio (i.e., 2.5 ml water to 1 g carrier).
- For Treatment A, the wet corn rootworm diet was mixed with Treatment 1 from the CEW bioassay, resulting in a 10% incorporation of the CEW diet by wet weight of the WCR diet.
- For Treatment B, the wet corn rootworm diet was mixed with Treatment 2 from the CEW bioassay, resulting in a 10% incorporation of the CEW diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C and 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete and mortality was assessed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from reporting.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 173.

F14. Evaluation of the Survival and Weight of Painted Lady Fed an Artificial Diet Containing Purified IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the PL bioassay consisted of Stonefly Heliothis diet.

The carrier for the WCR sensitive insect bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution consisted of ultrapure water.

The test dosing solution consisted of the test substance diluted in ultrapure water to achieve the concentration in the test diet (Treatment 2).

The heat-treated control dosing solution consisted of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes as described in section F14.a. Painted Lady IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was Vanessa cardui (painted lady; Lepidoptera: Nymphalidae; PL). PL was selected as a representative lepidopteran to characterize the spectrum of activity of IPD072Aa protein. PL eggs were obtained from Carolina Biological Supply Company (Burlington, NC, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was Diabrotica virgifera virgifera (western corn rootworm; Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

PL larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (targeting 2500 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained three replicates from each treatment. Each treatment was fed to a target of 30 PL individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between PL provided Treatments 1 and 2.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 4) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diet. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets and stability of the IPD072Aa protein dosing solution were also assessed. Stability of IPD072Aa protein in the carrier under bioassay conditions was confirmed under a separate Pioneer study and was not reassessed during this study. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein used in the PL test diet (Treatment 2).

Bias in the PL bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing and appropriate assay controls.

Diet Generation

Bulk dosing solutions for Treatments 1, 2, and 3 were prepared as described in Appendix A. The boric acid dosing solution for Treatment 4 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 2500 ng/mg diet wet weight. On each day of diet preparation, each dosing solution was mixed with carrier in a 3:1 ratio (i.e., 3 ml dosing solution to 1 g carrier), generating Treatments 1-4.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1, 2, and 3 were collected for characterization of diets as described in section F14.a. Painted Lady IPD072Aa Protein Bioassay Analytical Phase. The positive control diet (Treatment 4) was not characterized. A portion of Treatment 1 and 2 from each day of diet preparation were collected for use in diets for the sensitive insect bioassay as described in section F14.b. Painted Lady IPD072Aa Protein Sensitive Insect Bioassay.

PL Bioassay

A bioassay was conducted to determine the response of PL to IPD072Aa protein exposure via oral ingestion. PL eggs were incubated in an environmental chamber until the eggs hatched. PL neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One PL neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle for a total of 7 days. On Day 3, new bioassay plates were prepared as described for Day 0, and 600 µl of freshly prepared diet were dispensed to each well. Living PL larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparisons were made between PL fed diet containing IPD072Aa protein (Treatment 2) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of PL fed the artificial insect diet containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0$$

.

A significant difference was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption necessary for a two-sample *t*-test was not satisfied by data distributions of treatment groups 1 and 2; therefore, a non-parametric Wilcoxon two-sample test was conducted to test if exposure to artificial insect diet containing IPD072Aa protein caused growth inhibition. That is, to test if the weight of *PL* fed the artificial insect diet containing IPD072Aa protein (w_T) was less than the weight of individuals fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0$$

.

The equality of scale assumption was satisfied by a non-parametric Siegel-Tukey test; therefore, the Wilcoxon test is an effective test of the difference in medians. A significant difference was established if the P-value was < 0.05. SAS PROC NPAR1WAY was used to conduct both tests.

Demonstration of IPD072Aa Protein Activity in the PL Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 2 in the PL bioassay. Details regarding the sensitive insect bioassay are provided in section F14.b. Painted Lady IPD072Aa Protein Sensitive Insect Bioassay.

Results and Discussion

The PL bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 174.

The mortality of PL fed the test diet containing IPD072Aa protein (Treatment 2; 3 dead; 10 % mortality) was not significantly greater than those fed the bioassay control diet (Treatment 1; 5 dead; 16.7 % mortality; Fisher's exact test P-value = 0.8729), as shown in Table 174.

The mean weight of PL fed the test diet containing IPD072Aa protein (Treatment 2; 21.9 mg) was not significantly less than those fed the bioassay control diet (Treatment 1; 18.2 mg; Wilcoxon test P-value = 0.9138), as shown in Table 175.

Western blot analysis visually confirmed the dose and homogeneity of the IPD072Aa protein in the test diet (Treatment 2; Figure 66; Figure 67), as well as the stability of the IPD072Aa protein in the test dosing solution (Figure 68). Homogeneity assessment on one day of diet preparation is considered representative of both days of diet preparation. The IPD072Aa protein was detected in the heat-treated control diet (Treatment 3, Figure 66), but the band was less intense than that of the test diet (Treatment 2). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 66).

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 176. The WCR bioassay met the acceptability criterion (section F14.b. Painted Lady IPD072Aa Protein Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in the test diet used in the PL bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in the Sensitive Insect Bioassay section below).

Conclusion

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on survival or weight of PL.

Table 174. Summary Analysis of PL IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	30	5	16.7	0.8729
2	Test Diet	30	3	10.0	
3	Heat-treated Control Diet	30	3	10.0	--
4	Positive Control Diet	30	26	86.7	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

Table 175. Summary Analysis of PL IPD072Aa Protein Bioassay Weight Results

Treatment	Treatment Description	Number of Surviving Organisms	Mean ± Standard Deviation (mg)	Range (mg)	Wilcoxon Test P-Value
1	Bioassay Control Diet	25	18.2 ± 9.26	2.7 - 30.4	0.9138
2	Test Diet	27	21.9 ± 7.16	4 - 37.8	
3	Heat-Treated Control Diet	27	23.4 ± 6.68	5.8 - 30.9	--
4	Positive Control Diet	4	0.500 ± 0.141	0.4 - 0.7	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1000 ng/mg based on diet wet weight.

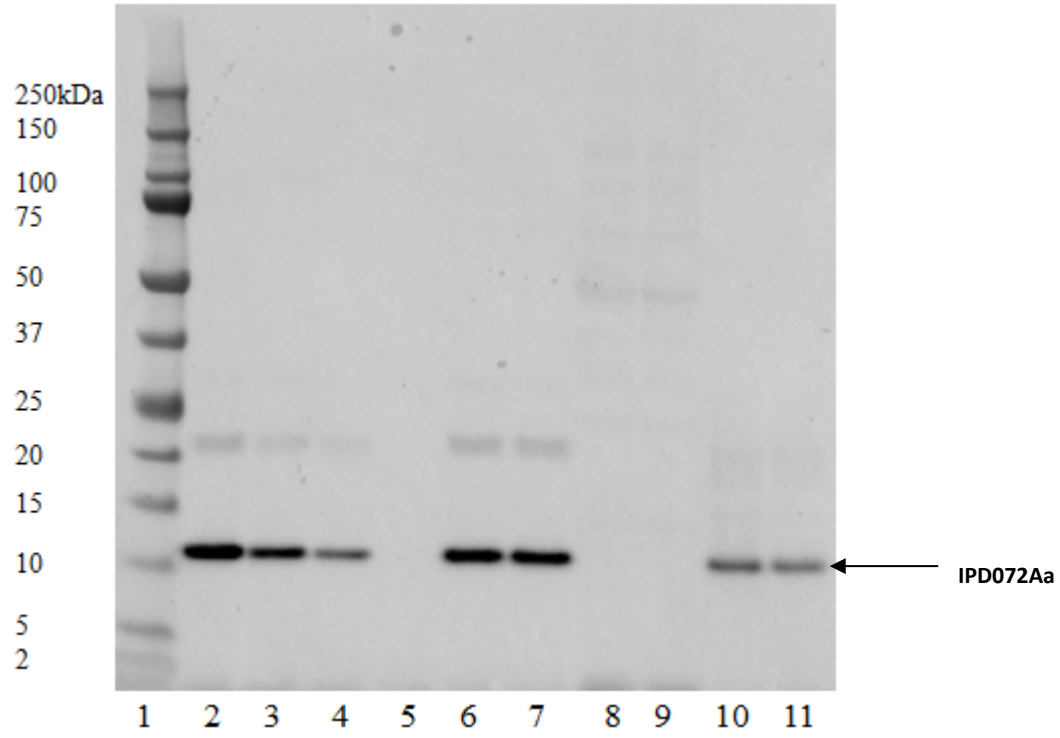
Table 176. Summary of PL IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0 ng IPD072Aa/mg	29[a]	13.8	25	0.484 ± 0.193	0.100 - 0.800
B	Test Diet	100 ng IPD072Aa/mg	28[a]	96.4	1	0.400[b]	NA

Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, in the PL bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the corn rootworm artificial diet. Not applicable (NA); there was only one surviving *WCR* in in the test diet group.

[a] Organisms counted as missing during the bioassay, or wells that contained more than one organism, were not included in the total number of observations for a given treatment.

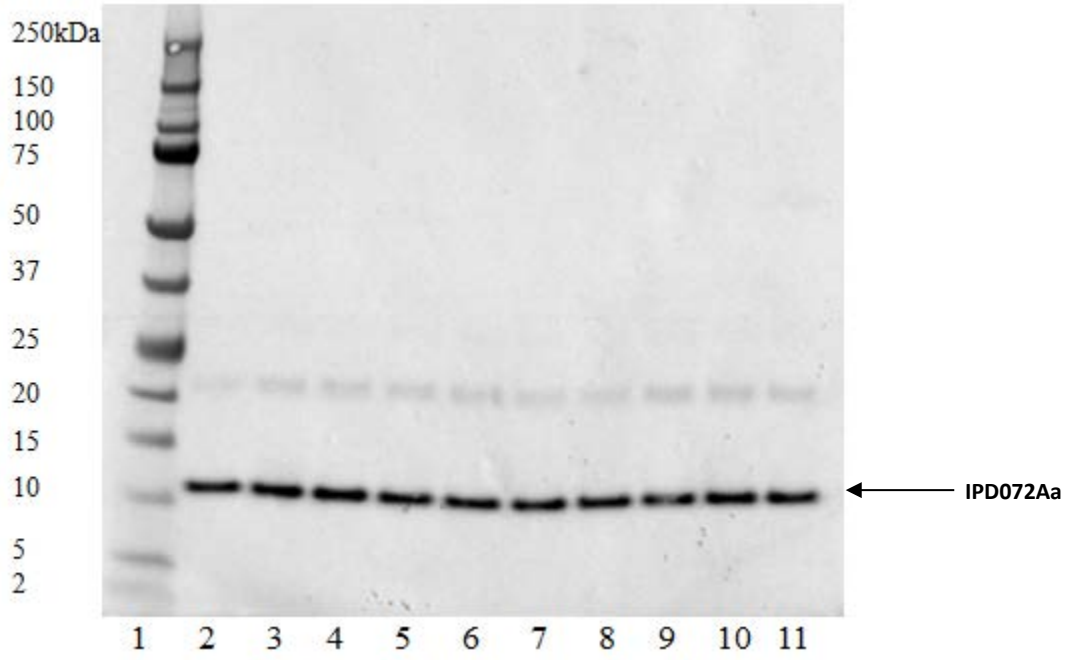
[b] The reported mean is the weight value of the one surviving larva after the 7-day feeding period; no standard deviation was calculated.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	Day 0 IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
7	Day 3 IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
8	Day 0 Bioassay Control Diet (Treatment 1; undiluted)
9	Day 3 Bioassay Control Diet (Treatment 1; undiluted)
10	Day 0 Heat-Treated Control Diet (Treatment 3; 10 ng)
11	Day 3 Heat-Treated Control Diet (Treatment 3; 10 ng)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lanes 8 and 9 are attributed to non-specific binding to diet components in the more concentrated bioassay control diet analytical samples (~130x relative to Lanes 6-7 and 10-11).

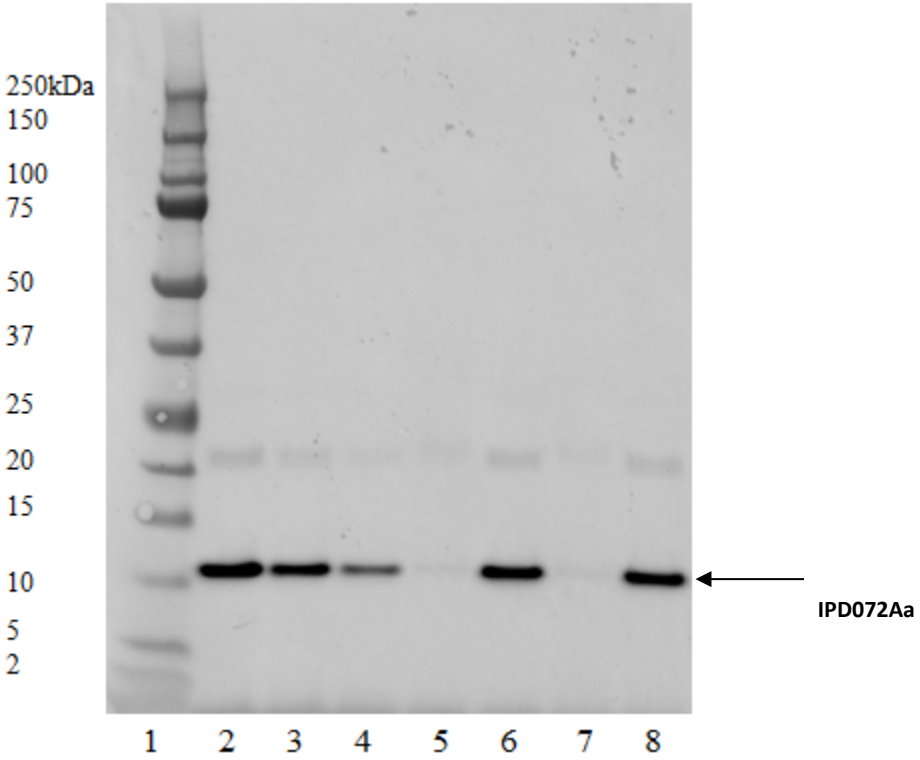
Figure 66. Dose Confirmation of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in the Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3), Day 0 and Day 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). Beginning, middle, and end refer to the stages in the diet distribution process at which the samples were collected.

Figure 67. Homogeneity Assessment of the IPD072Aa Protein in the Test Diet (Treatment 2)



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Dosing Solution (Day 0)
7	1X LDS Sample Buffer Blank
8	IPD072Aa Protein Dosing Solution (after final use)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test dosing solution used to prepare Treatment 2.

Figure 68. Stability Assessment of the IPD072Aa Protein Dosing Solution

F14.a. Painted Lady IPD072Aa Protein Bioassay Analytical Phase

The following dosing solutions were prepared for thePL bioassay:

Bioassay control dosing solution consisting of chilled ultrapure (American Society for Testing and Materials ASTM Type 1) water

Test dosing solution consisting of IPD072Aa protein test substance diluted in ultrapure water

Heat-treated IPD072Aa protein control dosing solution consisting of a portion of the test dosing solution that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose (1000 ng IPD072Aa protein per mg diet wet weight) and homogeneity of the IPD072Aa protein in the test diet (Treatment 2 in the PL bioassay). Homogeneity assessment on one day of diet preparation is considered representative of both days of diet preparation. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 3, respectively, in the PL bioassay), and the stability of the IPD072Aa protein in the test dosing solution were also assessed.

Preparation of Solutions

Dosing solutions were prepared on Day 0 of the PL bioassay and maintained chilled until use. To generate the test dosing solution, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to an IPD072Aa protein concentration of 1.33 mg/ml. To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution, samples of Treatments 1, 2, and 3 were collected as follows:

On Day 0, 15 samples of Treatment 2 were collected, five each from the beginning, middle, and end of the diet distribution process, to assess homogeneity of the IPD072Aa protein in the test diet. A sample collected for homogeneity was also used for visual confirmation of the dose of IPD072Aa protein in the Day 0 test diet.

On Day 3 of the diet distribution process, one sample of Treatment 2 was collected for visual confirmation of the dose of IPD072Aa protein in the Day 3 test diet.

On Day 0 and Day 3 of the diet distribution process, one sample each of Treatment 1 and Treatment 3 were collected to verify the presence or absence of immunodetectable IPD072Aa protein.

Sample Extraction and SDS-PAGE

Sub-samples of Treatments 1, 2, and 3 were weighed to approximately 40 mg each on wet ice in preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). In addition, an undiluted sub-sample of the test substance and sub-samples of the test dosing solution were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance or test dosing solution, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted as applicable, either before or after being heated at 90-100 °C for 5 minutes. Samples were then loaded into 4-12% Bis-Tris gels as follows:

For visual confirmation of the dose of IPD072Aa protein in the test diet and assessment of the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3 on Day 0 and Day 3, dilutions of the test substance (5, 10, and 20 ng) were loaded to a gel. Treatment 2 and Treatment 3 (Day 0 and Day 3; 10 ng each based on nominal concentration) were also loaded to the gel. Treatment 1 (Day 0 and Day 3) was loaded to the gel undiluted.

For homogeneity assessment, nine samples of Treatment 2 (three each from the beginning, middle, and end of the Day 0 diet distribution process) were loaded to a gel at 10 ng (based on nominal concentration of Treatment 2). A sample of the test substance (10 ng) was also loaded.

For stability assessment of the IPD072Aa protein dosing solution, samples of the test dosing solution (Day 0 and following final use) were loaded to a gel at 10 ng (based on nominal concentration of the test dosing solution). Dilutions of the test substance (5, 10, and 20 ng) were also loaded.

Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST once for 3-6 minutes each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Protein aggregation such as dimer formation may be observed; an IPD072Aa protein dimer would be expected to migrate at approximately 20 kDa.

F14.b. Painted Lady IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 2 used in the Vanessa cardui (PL) bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera virgifera

(WCR), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on Day 0 of the PL bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 10% Treatment 1 from the PL bioassay by wet weight of WCR diet)

Treatment B: Test Diet (containing 10% Treatment 2 from the PL bioassay by wet weight of diet; targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Day 0 and Day 3 diets prepared for the WCR bioassay contained a portion of Day 0 and Day 3 diets, respectively, prepared for the PL bioassay. Collected samples of Treatments 1 and 2 from each day of diet preparation for the PL bioassay were used to prepare Treatments A and B as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials (ASTM) Type 1) water was mixed with corn rootworm artificial diet at a 2.5:1 ratio (i.e., 2.5 ml water to 1 g carrier).

For Treatment A, the wet corn rootworm diet was mixed with Treatment 1 from the PL bioassay, resulting in a 10% incorporation of the PL diet by wet weight of the WCR diet.

For Treatment B, the wet corn rootworm diet was mixed with Treatment 2 from the PL bioassay, resulting in a 10% incorporation of the PL diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at

21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 3, new bioassay plates were prepared as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well, or wells containing more than one organism, were excluded from reporting.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 176 Weight data is summarized as means, standard deviations, and ranges and are presented inTable 176.

F15. Evaluation of the Survival and Weight of Codling Moth Fed an Artificial Diet Containing Purified IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubiliized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the CDM bioassay consisted of Stonefly Heliothis diet.

The carrier for the WCR bioassay consisted of an artificial insect diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution consisted of ultrapure water.

The test dosing solution consisted of the test substance diluted in ultrapure water to achieve the concentration in the test diet.

The heat-treated control dosing solution consisted of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes as detailed in section F15.a. CDM IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution consisted of boric acid (Sigma-Aldrich; H_3BO_3) and ultrapure water.

Test System

The test system was codling moth (Cydia pomonella, Lepidoptera: Tortricidae; CDM). CDM was selected as a representative lepidopteran to characterize the spectrum of activity of IPD072Aa protein. CDM eggs were obtained from Benzon Research Inc. (Carlisle, PA, USA) and identity was confirmed by study personnel.

The test system for the sensitive insect bioassay was western corn rootworm (Diabrotica virgifera virgifera, Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Experimental Design

CDM larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (targeting 5000 ng boric acid per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained three replicates from each treatment. Each treatment was fed to a target of 30 CDM individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and 24-hour dark cycle. Larvae were refed on Day 3 and missing and dead organisms were recorded. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Mortality and weight were statistically compared between CDM provided Treatments 1 and 2.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 4) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diet. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets and stability of the IPD072Aa protein dosing solution were also assessed. Stability of IPD072Aa protein in the carrier under bioassay conditions was confirmed under a separate Pioneer study and was not reassessed during this study. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in the CDM test diet.

Bias in the CDM bioassay and sensitive insect bioassay portions of this study was controlled through the randomization of treatments within blocks and the use of one or more control

diets. Bias in the characterization portion of the study was controlled through the use of replicate testing and appropriate assay controls.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Bulk dosing solutions for Treatments 1, 2, and 3 were prepared as described in Appendix A. The boric acid dosing solution for Treatment 4 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a target concentration of 5000 ng/mg diet wet weight. On each day of diet preparation, each dosing solution was mixed with carrier in a 3:1 ratio (i.e., 3 ml dosing solution to 1 g carrier) to generate Treatments 1, 2, 3, and 4.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1, 2, and 3 were collected for characterization of diets as described in section F15.a. CDM IPD072Aa Protein Bioassay Analytical Phase. The positive control diet (Treatment 4) was not characterized. A portion each of Treatment 1 and Treatment 2 were collected for use in the sensitive insect bioassay as described in section F15.b. CDM IPD072Aa Protein Sensitive Insect Bioassay.

CDM Bioassay

A bioassay was conducted to determine the response of CDM to IPD072Aa protein exposure via oral ingestion. CDM eggs were incubated in an environmental chamber until the eggs hatched. CDM neonates were used in the bioassay within 24 hours of hatching.

On Day 0, approximately 300 µl (i.e.,1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One CDM neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a 24-hour dark cycle. On Day 3, new bioassay plates were prepared as described for Day 0, living CDM larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as

missing from a well, or wells containing more than one organism, were excluded from reporting.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality and weight. Statistical comparison was made between CDM fed diet containing IPD072Aa protein (Treatment 2) and the bioassay control diet (Treatment 1) for the response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to compare if the mortality rate of CDM fed the artificial insect diet containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption was satisfied by data distributions of Treatment groups 1 and 2; therefore, a two-sample *t*-test was conducted to test if exposure to IPD072Aa protein caused growth inhibition. That is, to test if the weight of CDM fed the artificial insect diet containing IPD072Aa protein (w_T) was less than the weight of those fed the bioassay control diet (w_C). The corresponding hypothesis test was

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

The equality of variance assumption was satisfied by an F-test; therefore, the *t*-test based on pooled variance across treatment groups 1 and 2 was used. A significant difference was established if the

P-value was <0.05. SAS PROC TTEST was used to conduct the two-sample *t*-test.

Demonstration of IPD072Aa Protein Activity in the *CDM* Test Diet

A sensitive insect bioassay was performed using WCR larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 2 in the CDM bioassay. Details regarding the sensitive insect bioassay are provided in section F15.b. CDM IPD072Aa Protein Sensitive Insect Bioassay.

Results and Discussion

The CDM bioassay met the acceptability criteria as the combined dead and missing count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 177.

The mortality of CDM fed the test diet containing IPD072Aa protein (Treatment 2; 1 dead; 3.33% mortality) was not significantly greater than those fed the bioassay control diet (Treatment 1; 2 dead; 6.67% mortality; Fisher's exact test P-value = 0.8814), as shown in Table 177The mean weight of CDM fed the test diet containing IPD072Aa protein (Treatment 2; 5.58 mg) was not significantly less than those fed the bioassay control diet (Treatment 1; 5.65 mg; t-test P-value = 0.4263), as shown in Table 178.

Western blot analysis visually confirmed the dose and homogeneity of the IPD072Aa protein in the test diet (Treatment 2; Figure 69; Figure 70; Figure 71), as well as the stability of the IPD072Aa protein dosing solution (Figure 72). Homogeneity assessment on one day of diet preparation is considered representative of both days of diet preparation. The IPD072Aa protein was detected in the heat-treated control diet (Treatment 3, Figure 69; Figure 70), but the band was less intense than that of the test diet (Treatment 2). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 69; Figure 70).

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 3. The WCR bioassay met the acceptability criterion. The biological activity of the IPD072Aa protein in the test diet used in the CDM bioassay was demonstrated by 100% mortality of WCR fed the test diet (Treatment B; described in section F15.b. CDM IPD072Aa Protein Sensitive Insect Bioassay).

Conclusion

The results demonstrated exposure to a concentration of 1000 ng IPD072Aa protein per mg diet had no adverse effect on mortality or weight of CDM.

Table 177. Summary Analysis of CDM IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	30	2	6.67	--
2	Test Diet	30	1	3.33	0.8814
3	Heat-treated Control Diet	29[a]	1	3.45	--
4	Positive Control Diet	30	30	100	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1,000 ng/mg based on diet wet weight.
[a] Organisms counted as missing during the bioassay, or wells that contained more than one organism, were not included in the total number of observations for a given treatment.

Table 178. Summary Analysis of CDM IPD072Aa Protein Bioassay Weight Results

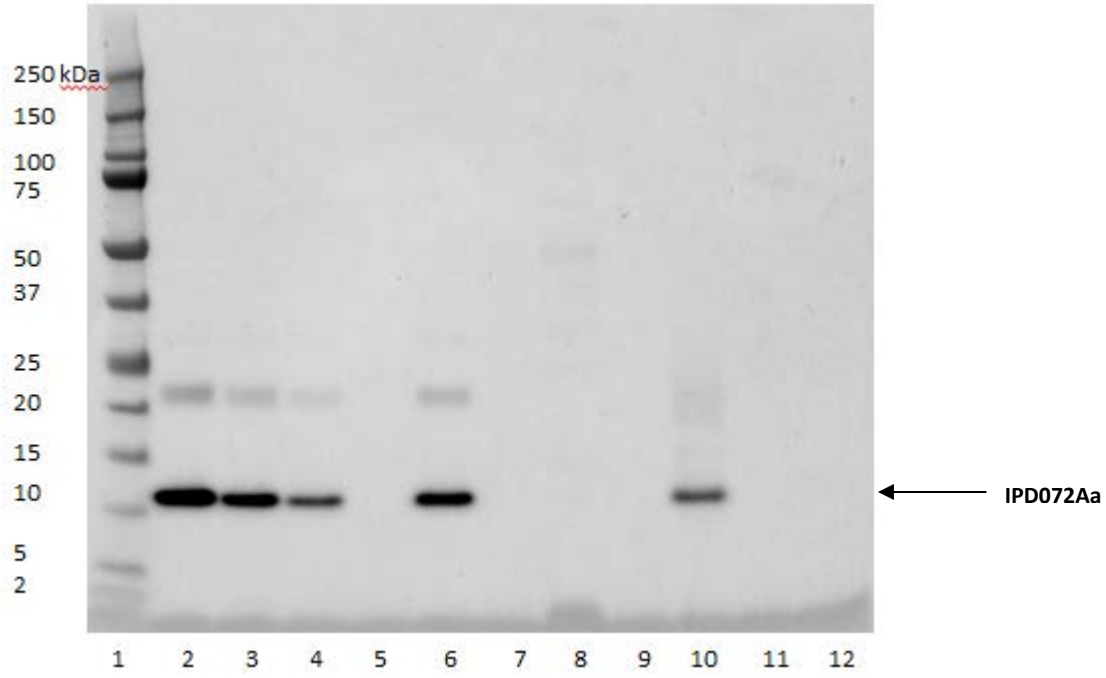
Treatment	Treatment Description	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	t-Test P-Value
1	Bioassay Control Diet	28	5.65 (5.20 - 6.09)	2.8 - 7.6	--
2	Test Diet	29	5.58 (5.05 - 6.12)	1.7 - 8.1	0.4263
3	Heat-Treated Control Diet	28	5.44 ± 1.57[a]	1.6 - 7.9	--
4	Positive Control Diet	0	NA	NA	--

Note: Treatment 2 targeted an IPD072Aa protein concentration of 1,000 ng/mg based on diet wet weight. Not applicable (NA); there were no surviving *Cydia pomonella* in the positive control diet group.
[a] Standard deviation is provided for mean values not subjected to a *t*-test.

Table 179. Summary of CDM IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0 ng IPD072Aa/mg	30	9	30.0
B	Test Diet	100 ng IPD072Aa/mg	30	30	100

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared from the same diet preparations used in Treatments 1 and 2, respectively, of the *Cydia pomonella* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the corn rootworm artificial diet.

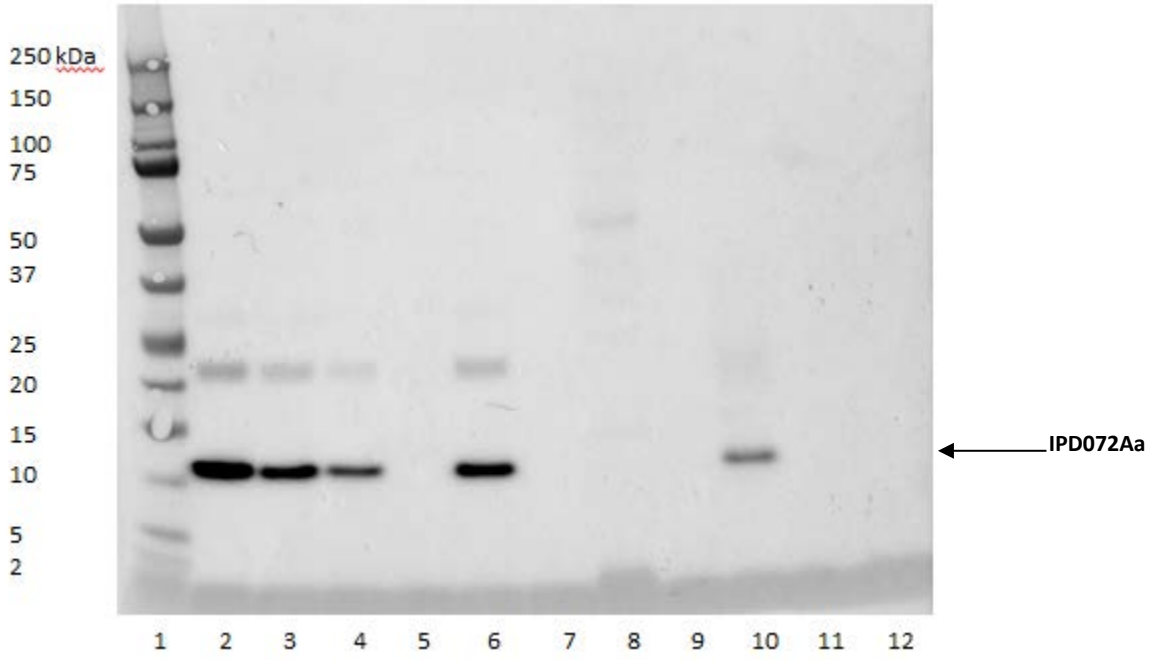


Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
7	1X LDS Sample Buffer Blank
8	Bioassay Control Diet (Treatment 1; undiluted)
9	1X LDS Sample Buffer Blank
10	Heat-Treated Control Diet (Treatment 3; 10 ng)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lane 8 are attributed to non-specific binding to diet components in the more concentrated bioassay control diet analytical sample (~130x relative to Lane 6 and Lane 10).

Figure 69. Dose Confirmation of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in the

Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3), Day 0

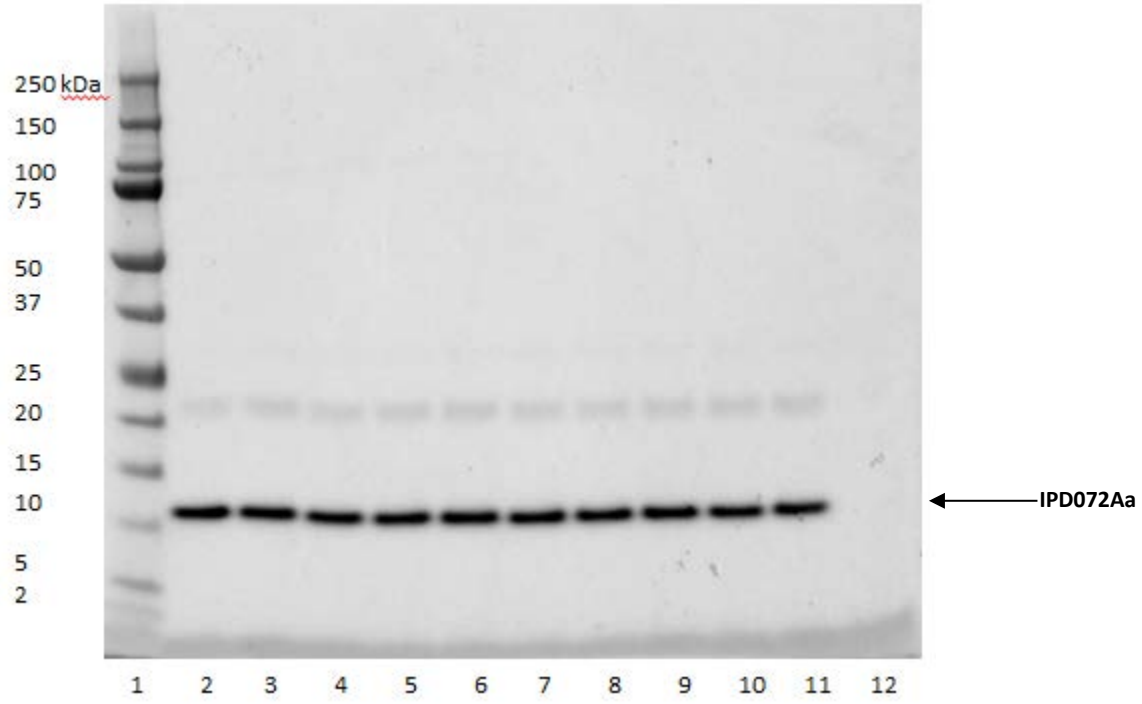


Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; 10 ng)
7	1X LDS Sample Buffer Blank
8	Bioassay Control Diet (Treatment 1; undiluted)
9	1X LDS Sample Buffer Blank
10	Heat-Treated Control Diet (Treatment 3; 10 ng)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. The test and heat-treated control diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). The faint higher molecular weight bands observed in Lane 8 are attributed to non-specific binding to diet components in the more concentrated bioassay control diet analytical sample (~130x relative to Lane 6 and Lane 10).

Figure 70. Dose Confirmation of the IPD072Aa Protein in the Test Diet (Treatment 2) and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in the

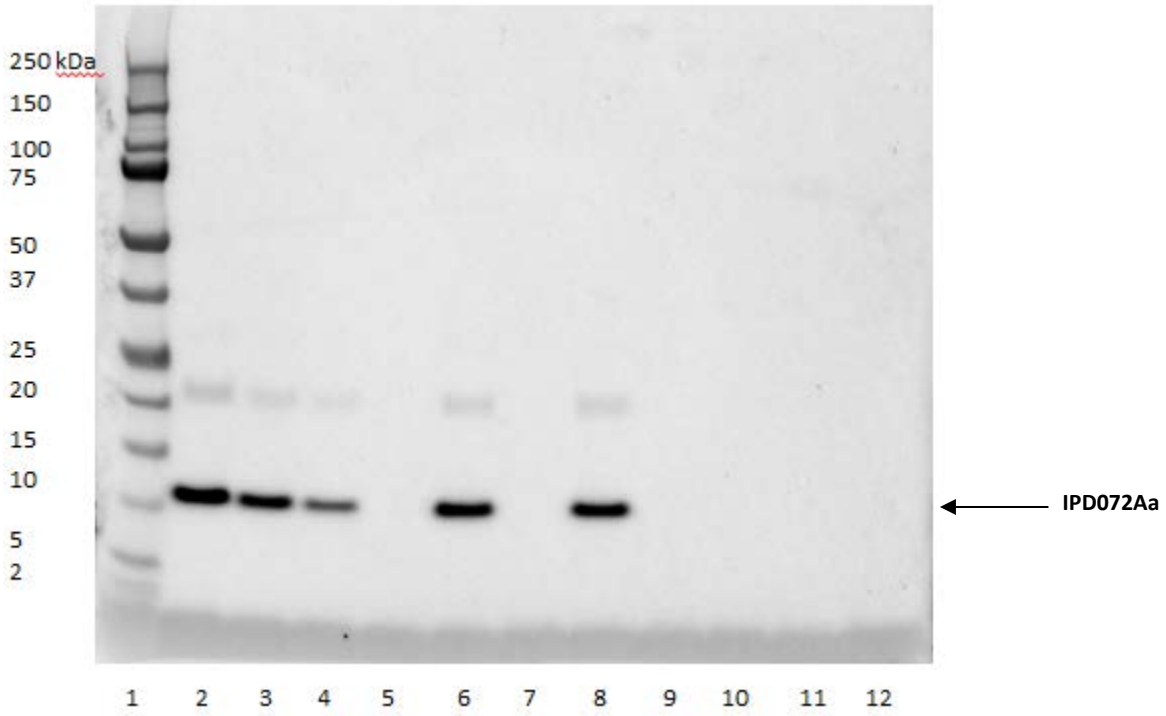
Bioassay Control Diet (Treatment 1) and Heat-Treated Control Diet (Treatment 3), Day 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test diet (Treatment 2). Beginning, middle, and end refer to the stage in the diet distribution process at which the samples were collected.

Figure 71. Homogeneity Assessment of the IPD072Aa Protein in the Test Diet (Treatment 2)



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Dosing Solution (Day 0)
7	1X LDS Sample Buffer Blank
8	IPD072Aa Protein Dosing Solution (Day 3)
9	1X LDS Sample Buffer Blank
10	1X LDS Sample Buffer Blank
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The faint band detected at approximately 20 kDa is attributed to IPD072Aa protein dimer formation. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Dosing solution samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the test dosing solution used to prepare Treatment 2.

Figure 72. Stability Assessment of the IPD072Aa Protein Test Dosing Solution

F15.a. CDM IPD072Aa Protein Bioassay Analytical Phase

The following dosing solutions were prepared for the CDM bioassay:

- Bioassay control dosing solution consisting of chilled ultrapure (defined as American Society for Testing and Materials ASTM Type 1) water
- Test dosing solution consisting of IPD072Aa protein test substance diluted in ultrapure water
- Heat-treated IPD072Aa protein control dosing solution consisting of a portion of the test dosing solution that was autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose (1000 ng IPD072Aa protein per mg diet wet weight) and homogeneity of the IPD072Aa protein in the test diet (Treatment 2 in the CDM bioassay). Homogeneity assessment on one day of diet preparation is considered representative of both days of diet preparation. The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 3, respectively, in the CDM bioassay), and the stability of the IPD072Aa protein dosing solution were also assessed.

Preparation of Solutions

Dosing solutions were prepared on Day 0 of the CDM bioassay and maintained chilled until use. To generate the test dosing solution, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to an IPD072Aa protein concentration of 1.33 mg/ml. To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet distribution for the CDM bioassay, samples of Treatments 1, 2, and 3 were collected as follows:

On Day 0, 15 samples of Treatment 2 were collected, five each from the beginning, middle, and end of the diet distribution process, to assess homogeneity of the IPD072Aa protein in the test

diet. One of the samples collected for homogeneity was also used for visual confirmation of the dose of IPD072Aa protein in the Day 0 test diet.

On Day 3, one sample of Treatment 2 was collected for visual confirmation of the dose of IPD072Aa protein in the test diet.

On Day 0 and Day 3, a sample each of Treatment 1 and Treatment 3 were collected to verify the presence or absence of immunodetectable IPD072Aa protein.

Sample Extraction and SDS-PAGE

Sub-samples of Treatments 1, 2, and 3 were weighed to approximately 40 mg each on wet ice in preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). In addition, undiluted sub-samples of the test substance and sub-samples of the test dosing solution were prepared for SDS-PAGE by combining 65% IPD072Aa test substance or test dosing solution, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80°C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as follows:

For visual confirmation of the dose of IPD072Aa protein in the test diet and assessment of the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3, dilutions of the test substance were loaded (5, 10, and 20 ng) to gels (one gel for Day 0 samples and one gel for Day 3 samples). Treatment 2 and Treatment 3 (Day 0 and Day 3; 10 ng each based on nominal concentration) were also loaded to each gel. Treatment 1 (Day 0 and Day 3) was loaded to each gel undiluted.

For homogeneity assessment, nine samples of Treatment 2 (three each from the beginning, middle, and end of the Day 0 diet distribution process) and a sample of the test substance were loaded to a gel at 10 ng each.

For stability assessment of the IPD072Aa dosing solution, samples of the test dosing solution (Day 0 and following final use) were loaded to a gel at 10 ng (based on nominal concentration of the test dosing solution). Dilutions of the test substance (5, 10, and 20 ng) were also loaded.

Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline containing polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Protein aggregation such as dimer formation may be observed; an IPD072Aa protein dimer would be expected to migrate at approximately 20 kDa.

F15.b. CDM IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 2 used in the Cydia pomonella (CDM) bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera

virgifera (WCR), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on Day 0 of the CDM bioassay.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing 10% Treatment 1 from the CDM bioassay by wet weight of WCR diet)

Treatment B: Test Diet (containing 10% Treatment 2 from the CDM bioassay by wet weight of diet; targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Day 0 and Day 3 diets prepared for the WCR bioassay contained a portion of Day 0 and Day 3 diets, respectively, prepared for the CDM bioassay. Collected samples of Treatments 1 and 2 from each of the two days of diet preparation for the CDM bioassay were used to prepare Treatments A and B as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials (ASTM) Type 1) water was mixed with corn rootworm artificial diet at a 2.5:1 ratio (i.e., 2.5 ml water to 1 g carrier).

For Treatment A, the wet corn rootworm diet was mixed with Treatment 1 from the CDM bioassay, resulting in a 10% incorporation of the CDM diet by wet weight of the WCR diet.

For Treatment B, the wet corn rootworm diet was mixed with Treatment 2 from the CDM bioassay, resulting in a 10% incorporation of the CDM diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e.,1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each

bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and 24-hour dark cycle for 7 days. On Day 3, new bioassay plates were prepared as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete and mortality was assessed.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay and multiplied by 100 and are presented in Table 179.

Appendix G. IPD072Aa Protein Non-Target Organism Bioassay Materials and Methods

G1. Evaluation of the Survival and Reproduction of Springtail Fed an Artificial Diet Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of ground and autoclaved Saccharomyces cerevisiae (Type II) yeast.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solution used to prepare Treatment 2 consisted of the test substance diluted in ultrapure water to achieve the concentration in the test diet.

The heat-treated control dosing solution used to prepare Treatment 3 consisted of a portion of the test dosing solution used to prepare Treatment 2 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section G1.a. Springtail IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution used to prepare Treatment 4 consisted of teflubenzuron ($C_{14}H_6Cl_2F_4N_2O_2$) and ethanol.

Test System

The test system was Folsomia candida (springtail; Collembola: Isotomidae). Springtail was selected as a representative non-target organism to characterize IPD072Aa protein. Springtail adults were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

Springtail were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Heat-Treated Control Diet (targeting 500 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 4: Positive Control Diet (targeting 1000 ng teflubenzuron per mg diet dry weight)

Treatments were arranged in a generalized randomized block design with a total of 2 blocks. Each block consisted of 16 small, wide-mouth glass jars (four jars per treatment) with screw top lids vented for aeration. Each jar contained a target of 10 adult organisms and each treatment was fed to a target of 80 Springtail individuals. The bioassay was conducted in an environmental chamber set at 20 °C, 65% relative humidity, and continuous dark. Springtail were refed daily. After 28 days, the bioassay was complete, adult mortality was assessed, and adult and juvenile organisms were each counted.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- Adult mortality exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mean offspring per bioassay vessel is less than 100 individuals for Treatment 1.
- The coefficient of variation (CV) for mean reproduction is greater than 30% for Treatment 1.
- The mortality of the positive control diet (Treatment 4) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose, homogeneity, and frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3 was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of the IPD072Aa protein in Treatment 2.

Bias in the Springtail bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-3 were prepared and maintained as described in Appendix A. The positive control dosing solution for Treatment 4 was prepared by solubilizing and diluting teflubenzuron in ethanol to achieve a nominal concentration of 1000 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 2:1 ratio (i.e., 2 ml dosing solution to 1 g carrier) in 50-ml tubes to generate Treatments 1-4. After mixing, diets were lyophilized, aliquoted into individual storage tubes, and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1-3 were collected for characterization of diets as described in Appendix A. In addition, a portion each of Treatment 1 and Treatment 2 was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in section G1.b. Springtail IPD072Aa Protein Sensitive Insect Bioassay.

The positive control diet (Treatment 4) was not characterized.

Springtail Bioassay

A bioassay was conducted to determine the response of Springtail to IPD072Aa protein exposure via oral ingestion.

On Day 0, diet aliquots were removed from storage and glass jars were prepared with a substrate mixture containing 47% Plaster of Paris, 6% charcoal, and 47% deionized water. Bioassay jars were labeled by treatment and replicate and ten, 11-day-old, Springtail adults were placed in each jar. Diets were distributed to small squares of weigh paper by treatment, a weigh paper square was placed in each infested bioassay jar, and the jars were loosely capped. The bioassay was conducted in an environmental chamber set at 20 °C, 65% relative humidity, and continuous dark. Every day during the bioassay, infested jars were removed from the environmental chamber, diet was replaced with new aliquots as described for Day 0, dry substrate was re-saturated with deionized water as necessary, and the bioassay jars were loosely capped and returned to the environmental chamber. After 28 days, the bioassay was complete, adult mortality was assessed, and adult and juvenile organisms were each counted.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and reproduction. Statistical comparisons were made between Springtail fed diet containing IPD072Aa protein (Treatment 2) and the bioassay control diet (Treatment 1) for the response variables.

Mortality

Statistical analysis was conducted using Fisher's exact test to compare if the mortality rate of Springtail adults fed the artificial insect diet containing IPD072Aa protein (m_T) was higher than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test is

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

Significance was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct Fisher's exact test.

Reproduction

A generalized linear mixed model was fit to the reproduction data assuming a Poisson distribution of the number of offspring, a log link function, and the Laplace method of integral approximation. Treatment was modeled as a fixed effect. Jar number within each treatment and block was considered a random effect.

The estimated model was used to test if the reproduction from the *Springtail* fed the artificial insect diet containing IPD072Aa protein (w_T) was less than the reproduction from those fed the bioassay control diet (w_C). The corresponding hypothesis test is

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0 .$$

SAS PROC GLIMMIX was utilized for generalized linear mixed model analysis, and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means. A significant difference was identified if the P-value was < 0.05.

Demonstration of IPD072Aa Protein Activity in the Springtail Test Diet

A sensitive insect bioassay was performed using *WCR* larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 2 in the Springtail bioassay. A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive

insect bioassay. Details regarding the sensitive insect bioassay are provided in section G1.b. Springtail IPD072Aa Protein Sensitive Insect Bioassay.

The Springtail bioassay met the acceptability criteria as adult mortality in the bioassay control diet (Treatment 1) group did not exceed 20%, the mean offspring count in Treatment 1 was ≥ 100, the CV for mean reproduction in Treatment 1 was ≤ 30%, and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 180.

The mortality of Springtail fed the test diet containing 500 ng/mg IPD072Aa protein (Treatment 2; 2.50%; P-value = 0.2453) was not significantly greater than those fed the bioassay control diet (Treatment 1), as shown in Table 180.

The mean reproduction of Springtail fed Treatment 2 (394 offspring, P-value=0.0344) was significantly less than the mean reproduction of those fed Treatment 1 (460 offspring), as shown in Table 181. While this endpoint was statistically significantly different, this result was not considered to be biologically relevant, given that reproduction in Treatment 2 greatly exceeded the collembola OECD reproduction criteria of 100 mean offspring (OECD, 2009) and the overlapping range of offspring observed between Treatments 1, 2 and 3.

Western blot analysis visually confirmed the dose, homogeneity, and frozen storage stability of the IPD072Aa protein in Treatment 2 (Figure 73; Figure 74; Figure 75). The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 3 was also assessed (Figure 73). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 3; Figure 73); however, the band was less intense than that of the test diet (Treatment 2). No IPD072Aa protein was detected in the bioassay control diet (Treatment 1; Figure 73).

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 182 and Table 183. The WCR bioassay met the acceptability criterion (G1.b. Springtail IPD072Aa Protein Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in Treatment 2 of the Springtail bioassay was demonstrated by increased mortality and decreased weight for WCR fed the test diet (Treatment B) compared to those fed the bioassay control diet (Treatment A). The mortality of WCR fed Treatment B (32.1% mortality; P-value = 0.0048) was significantly greater than those fed Treatment A (3.33%), as shown in Table 3. WCR fed Treatment B (median weight 0.1 mg; Wilcoxon test P-value <0.0001) had significantly greater probability to weigh less or more than those fed Treatment A (median weight 0.5 mg), as shown in Table 4. The Siegel-Tukey test (P-value = 0.1211 for Treatment B) did not show significant evidence that the scales of the populations differed, as shown in Table 183.

Conclusion

The results demonstrated exposure to a concentration of 500 ng IPD072Aa protein per mg diet (Treatment 2) had no adverse effect on survival of Springtail. No statistically significant differences were observed in mortality between Springtail fed Treatment 2 (2.50%) and those fed the bioassay control diet (Treatment 1; 0%). While a statistically significant difference was observed in mean reproduction between Springtail fed Treatment 2 (394 offspring) and those fed Treatment 1 (460 offspring), this result was not considered to be of biological relevance, based on acceptability guidelines for collembolan reproduction established by the OECD (2009), as well as the overlapping range of offspring produced in individual replicates within Treatments 1, 2, and 3.

Table 180. Summary Analysis of Springtail IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	81[a]	0	0.00	--
2	Test Diet	500	80	2	2.50	0.2453
3	Heat-treated Control Diet	500	80	1	1.25	--
4	Positive Control Diet	0[b]	80	74	92.5	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet dry weight.
[a] One jar for Treatment 1 contained 11 organisms.
[b] Treatment 4 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

Table 181. Summary Analysis of Springtail IPD072Aa Protein Bioassay Reproduction Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Mean Number of Offspring Per Jar (95% Confidence Interval)	Range	%CV	P-Value
1	Bioassay Control Diet	0	460 (409 - 517)	356 - 571	15.7	--
2	Test Diet	500	394 (350 - 444)	317 - 554	18.5	0.0344[a]
3	Heat-treated Control Diet	500	420 ± 94.8[b]	269 - 599	--	--
4	Positive Control Diet	0[c]	0	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet dry weight. Not applicable (NA); there were no offspring recorded for *Folsomia candida* in Treatment 4.
[a] A statistically significant difference (P-value < 0.05) was observed.
[b] Standard deviation is provided for mean values not subjected to linear mixed model analysis.
[c] Treatment 4 contained a targeted concentration of 1000 ng teflubenzuron per mg diet dry weight.

Table 182. Summary Analysis of Springtail IPD072Aa Protein Sensitive Insect Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
A	Bioassay Control Diet	0	30	1	3.33	--
B	Test Diet	28.5	28[a]	9	32.1	0.0048[b]

Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 2, respectively, of the Springtail bioassay. The concentration of IPD072Aa protein in Treatment B was based on wet weight of the artificial diet.

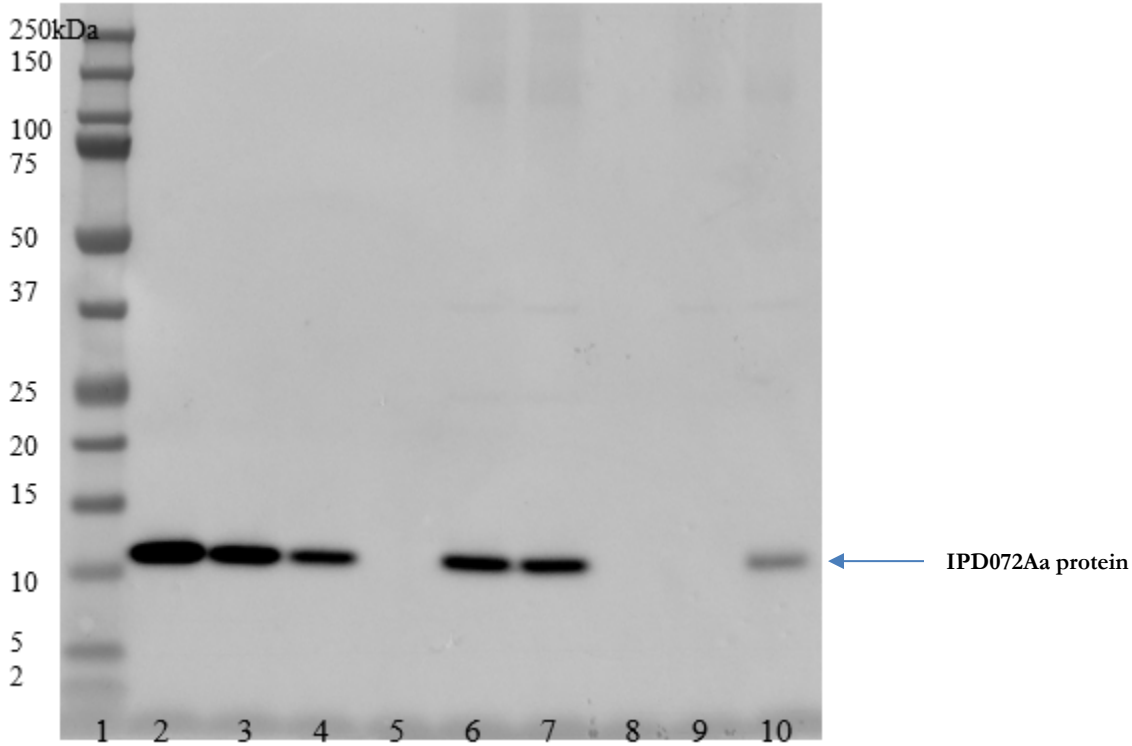
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] A statistically significant difference (P-value < 0.05) was observed.

Table 183. Summary Analysis of Springtail IPD072Aa Protein Bioassay Sensitive Insect Bioassay Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Median (mg)	Mean ± Standard Deviation (mg)	Range (mg)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
A	Bioassay Control Diet	0	29	0.5	0.517 ± 0.126	0.1 - 0.7	--	--
B	Test Diet	28.5	19	0.1	0.126 ± 0.0562	0 - 0.2	<0.0001[a]	0.1211

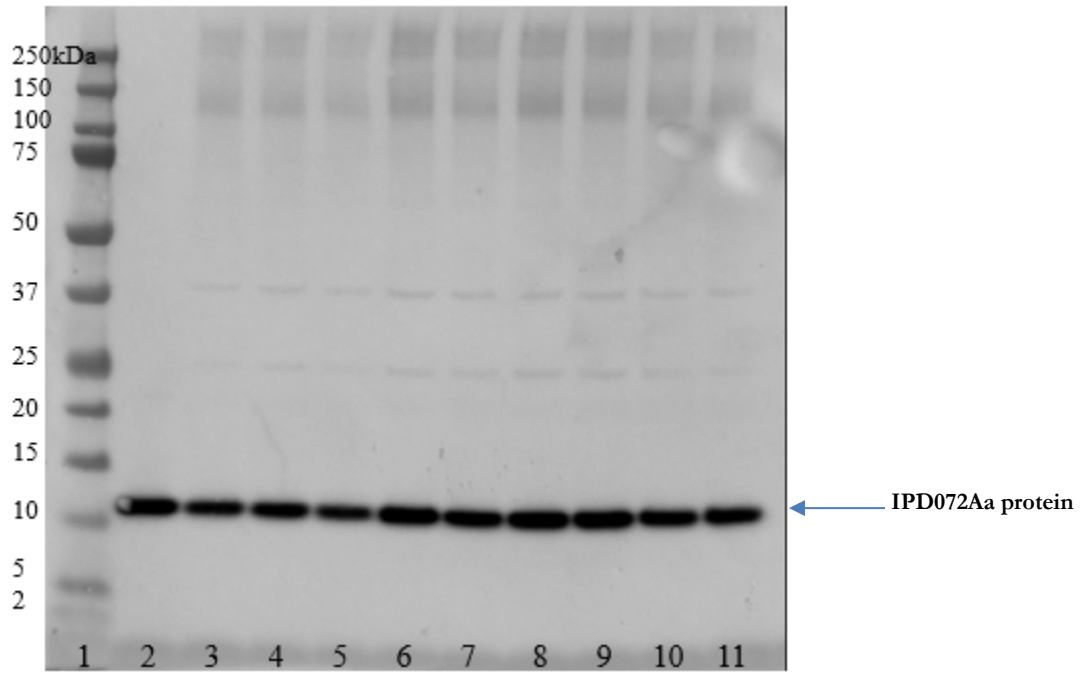
[a] A statistically significant difference (P-value < 0.05) was observed.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2; Rep 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Rep 2)
8	1X LDS Sample Buffer Blank
9	Bioassay Control Diet (Treatment 1)
10	Heat-Treated Control Diet (Treatment 3)

Note: Nanogram (ng) and lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatment 2 was loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein. Treatment 3 was loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2.

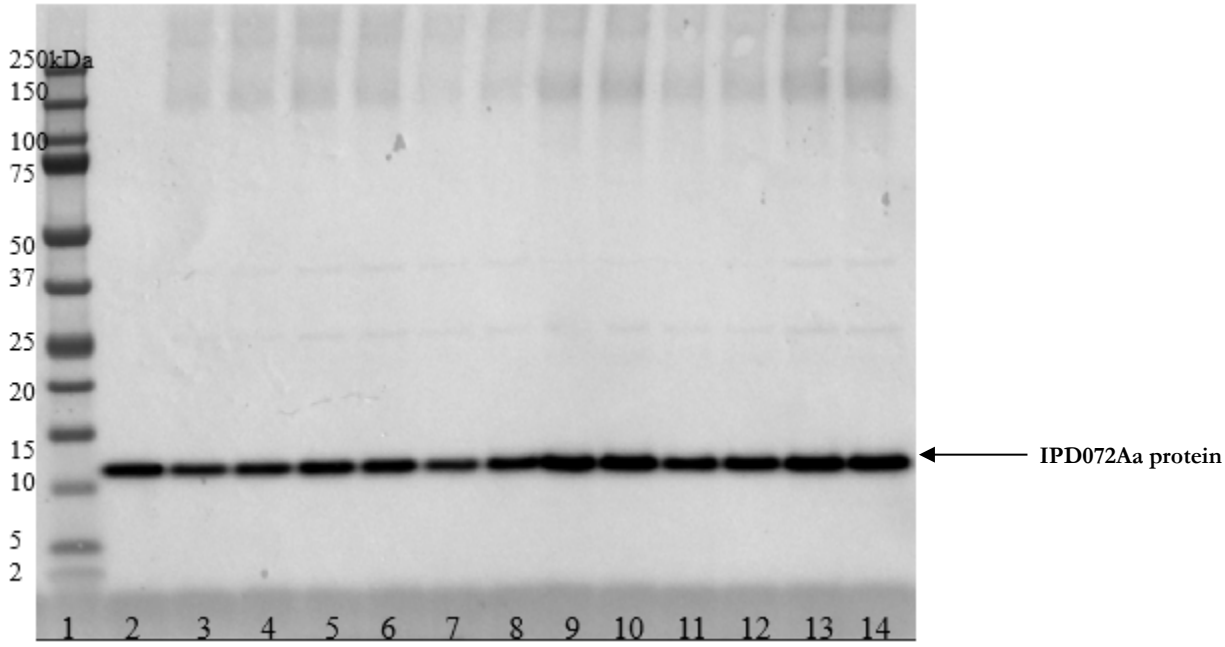
Figure 73. Dose Confirmation of the IPD072Aa Protein in Treatment 2 and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)

Note: Nanogram (ng). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

Figure 74. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
6	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Week 2)
8	IPD072Aa Protein Test Diet (Treatment 2; Week 2)
9	IPD072Aa Protein Test Diet (Treatment 2; Week 3)
10	IPD072Aa Protein Test Diet (Treatment 2; Week 3)
11	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
12	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
13	IPD072Aa Protein Test Diet (Treatment 2; Week 5)
14	IPD072Aa Protein Test Diet (Treatment 2; Week 5)

Note: Nanogram (ng). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Weeks 1-5 refer to the approximate length of time samples were stored frozen (-80 °C freezer unit) prior to analysis.

Figure 75. Frozen Storage Stability Assessment of the IPD072Aa Protein in Treatment 2

G1.a. Springtail IPD072Aa Protein Bioassay Analytical Phase

The following dosing solutions were prepared for the Springtail bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solution used to prepare Treatment 2 consisting of IPD072Aa protein test substance diluted in ultrapure water to achieve the concentration in the test diet

Heat-treated IPD072Aa protein control dosing solution used to prepare Treatment 3 consisting of a portion of the test dosing solution used to prepare Treatment 2 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose, homogeneity, and frozen storage stability of the IPD072Aa protein in Treatment 2 (500 ng IPD072Aa protein per mg diet dry weight). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 3, respectively) was also assessed.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation. To generate the test dosing solution for Treatment 2, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.250 mg/ml). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the Springtail bioassay, samples were collected as shown in Table 184.

Table 184. Springtail IPD072Aa Protein Bioassay Diet Samples

Treatment	Number of Samples	Analysis
2	5 beginning	Homogeneity of IPD072Aa protein
	5 middle	
	5 end	
2	NA	Visual confirmation of IPD072Aa protein dose
1	1 from each treatment	Verify presence or absence of immunodetectable IPD072Aa protein
3		
2	1 bulk sample[a]	Frozen storage stability (-80 °C freezer unit)

Note: Not applicable (NA); homogeneity samples were used for this assessment.

[a] Two sub-samples were collected at each time point (Weeks 1-5); homogeneity samples were used for Day 0 assessment.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1-3 were weighed to approximately 10 mg on dry ice in preparation for SDS-PAGE. Sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 5 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown in Table 185.

Table 185. Springtail IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment	Number of Samples
Dose confirmation of IPD072Aa protein	2	2
Homogeneity	2	3 beginning
		3 middle
		3 end
Frozen storage stability	2	2 per timepoint (Day 0, Weeks 1-5)
Presence/absence of IPD072Aa protein	1	1
	3	1

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before and after the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST four times for at least 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

G1.b. Springtail IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 2 used in the Springtail bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera virgifera (WCR), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated on the final day of the Springtail bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)

Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 28.5 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 2 during diet aliquoting for the Springtail bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the Springtail bioassay, resulting in a 20% incorporation of the Springtail diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 2 from the Springtail bioassay, resulting in a 20% incorporation of the Springtail diet by dry weight of the WCR diet.

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

The response variables of interest were mortality and weight. Statistical comparisons were made between WCR fed diet containing IPD072Aa protein (Treatment B) and the bioassay control diet (Treatment A) for the response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

WCR fed the artificial insect diet containing IPD072Aa protein (m_T) was different from the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0\colon m_T - m_c = 0 \quad vs. \quad H_a\colon m_T - m_c \neq 0.$$

A significant difference was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

The normality assumption necessary for a two-sample *t*-test was not satisfied by data distributions of treatment groups A and B; therefore, non-parametric two-sample tests were conducted to test if exposure to IPD072Aa protein caused weight difference. The corresponding hypothesis test was

$$H_0\colon F_T(x) = F_c(x) \;\; vs. \;\; H_a\colon F_T \neq F_c.$$

Where $F_T(x)$ is the cumulative distribution of the weight of *WCR* fed the artificial insect diet containing IPD072Aa protein, and $F_c(x)$is the cumulative distribution of the weight of individuals fed the bioassay control diet. Therefore, significance would indicate that insects fed the diet containing IPD072Aa protein would have a greater probability to weigh less or more than those fed the control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992).

The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

A summary analysis of WCR bioassay mortality results can be found in Table 182. A summary analysis of WCR bioassay weight results can be found in Table 183.

G2. IPD072Aa Protein: Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure

The procedures used in this study are based on an OECD Guidance Document No. 239 dated 15 July 2016 (OECD, 2016), procedures discussed in Protocol for the in vitro rearing of honey bee (*Apis mellifera* L.) workers (Schmehl, et. al., 2016), and recent input from the U.S. EPA.

Test Substance

The test substance consisted of IPD072Aa protein. Stability was certified under -80 °C freezer unit storage condition.

The IPD072Aa protein working stock solution was received in solution on 24 May 2018 from Pioneer Hi-Bred International, Inc., Johnston, Iowa.

Upon receipt at the contract research facility (CRO) the IPD072Aa protein Working Stock Solution (No. 9445) was stored in a freezer (-80 °C) in the original container.

Test Organism

The honey bee larvae (*Apis mellifera*) used to initiate the definitive test were provided by Wood's Beekeeping Supply, Lincoln, Rhode Island. The source hives are identified as Italian hybrids as they contain a mixture of the original subspecies, *A. mellifera ligustica*, and unidentified subspecies which commonly co-exist in commercial bee hives. The queen from three or more hives was isolated on a single frame for one day to provide known-aged eggs and subsequent larvae. The frames were delivered to the CRO so that ≤24-hour old larvae (hatched within the previous 24 hours) were removed for testing. The hives from which the larvae were obtained were not previously exposed to any chemical treatments within four weeks of test initiation.

Culture and Test Vessels

The larval culture and test vessels were sterile, 48-well cell culture plates (1.6 mL/well; Corning) containing a plastic queen cup grafting cell (Mann Lake) in 32 wells during acclimation and in 18 wells during exposure. Each plate was labeled with the treatment or control, replicate, and study number. The perimeter wells within each plate not containing larvae were partially filled with deionized water to assist in maintaining the relative humidity at >90%.

The pupation plates were sterile, 24-well cell culture plates (3.4 mL/well; Corning) each containing two layers of sterilized dust-free Kimwipes.

Transfer of Larvae

Frames containing the isolated brood cells were removed from each hive, adult bees were removed from each frame, and the frames were then brought into the laboratory. Frames containing newly hatched larvae were placed in a grafting chamber maintained at approximately 26 to 33 °C. The larvae were removed from brood cells by placing a grafting tool under the larvae and carefully lifting it from its brood cell. To reduce the potential for contamination of pathogenic microorganisms, the transfer tool was sanitized with 70% ethyl alcohol after each transfer and blotted dry before use with the next larva. Care was taken to keep the larvae oriented to allow their spiracles access to air for respiration. Excess larvae were collected on day 1 to ensure enough were available for testing on day 3.

Acclimation Phase

The initial phase of the test involved a 2-day acclimation phase. Diets were prepared as described below. Larvae were fed 20 µL of untreated diet A on the day of transfer into the cell plates (day 1) and not fed on day 2. After the 2-day acclimation phase, dead or discolored larvae contained in a grafting cell were removed from the culture plates. Remaining healthy larvae were randomly assigned to cell plates using computer generated random numbers until each treatment contained 12 larvae from three different hives for a total of 36 larvae per test group evenly divided between two plates (i.e., 18 larvae per plate) and placed in an environmental chamber.

Test Concentration Selection

Based on consultation with Pioneer, target daily diet concentrations of 0.025 and 0.050 µg/a.i./larva/day were used to calculate nominal cumulative doses of 0.10 and 0.20 µg a.i./larva. These nominal cumulative doses and a control were selected for the definitive exposure.

Diet Preparation

The deionized water used to prepare royal jelly diet was sterilized before use. Three untreated diet types were prepared prior to test initiation. The diets were prepared as follows:

Component	Diet A[a]	Diet B[b]	Diet C[c]
Deionized water (g)	800	990	780
D-glucose[d] (g)	95	150	230
D-fructose[d] (g)	95	150	230
Yeast extract[de] (g)	16	30	52
Royal jelly[f] (g)	800	990	1300

[a] Diet fed on day 1.
[b] Used for treated diet fed on exposure day 3.
[c] Used for treated diet fed on exposure days 4, 5, and 6.
[d] Supplier: Sigma Aldrich, Saint Louis, Missouri.
[e] Yeast extract is made from *Saccharomyces cerevisiae* species of yeast.
[f] Supplier: Stakich, Inc., Troy, Michigan.

NOTE: Royal jelly diet preparation ratios are based on modifications from Schmehl et al. (2016).

On feeding days, an aliquot of each diet was brought to test temperature by placing within the test incubator before being added to the plate wells. Thawed, untreated, and treated diet including unused diet was stored in a freezer when not in use. All diets were appropriately disposed of after final feeding.

Representative samples of the royal jelly diet, comprised of water and royal jelly and additional ingredients, were analyzed periodically for the presence of PCBs, and toxic metals by Eurofins Lancaster Laboratories Environmental, Lancaster Pennsylvania using U.S. EPA standard methods (US-EPA, 1997). Additionally, a sample of royal jelly diet was analyzed for common pesticides and antibiotics by the U.S. Department of Agriculture (USDA), Gastonia, North Carolina. The diet was considered to be of acceptable quality since no analytes were measured at concentrations considered to be toxic to the test organisms (ASTM, 2007).

Preparation of Diets

Test Substance

A 0.55 mg a.i./mL working stock solution was prepared by Pioneer Hi-Bred, International for incorporation into royal jelly diet. An aliquot of stock solution was removed from storage and thawed at 4 ± 3 °C in order to dose diets used on days 3, 4, 5, and 6. Due to the different amount of diets offered to the test organisms and to provide a fixed daily dose, the diet concentrations were different on each exposure day as indicated in the table below. A measured amount of stock solution was added to a batch of the appropriate diet.

Treated diets were prepared individually using the following preparation scheme:

Test Day	Dosing Stock Concentration (mg a.i./mL or µg a.i./µL)	Volume of Dosing Stock Solution Used (µL)	Total Weight of Diet Used (g)	Nominal Diet Concentration (µg a.i./mL diet)	Total Amount Diet Dispensed per Well[a]		Nominal Daily Diet Concentration (µg a.i./larva/day)	Nominal Cumulative Dose (µg a.i./larva)
					(µL)	(g)		
3	0.55	19.4	10	1.25	20	0.023	0.025	0.10
4	0.55	12.9	10	0.833	30	0.035		
5	0.55	9.70	10	0.625	40	0.047		
6	0.55	7.76	10	0.500	50	0.058		
3	0.55	38.8	10	2.5	20	0.023	0.050	0.20
4	0.55	25.9	10	1.67	30	0.035		
5	0.55	19.4	10	1.25	40	0.047		
6	0.55	15.5	10	1.00	50	0.058		

[a] The weighted density is based on proportions of each diet type (B and C) within the total amount of diet dispensed per well (µL) as would be used in larval chronic testing. The weighted density is 1.1710 g/mL.

NOTE: Resulting diet concentrations and nominal dosages were calculated using the actual (unrounded) results and not the rounded values presented in this table.

All treated royal jelly diets appeared opaque and light yellow in color with no visible undissolved test substance after preparation. Untreated diet was used for the control.

Reference Toxicant

In order to assess the health of the hives used to provide the test population, an 8-day reference test was included in the test design and was conducted concurrently with the definitive exposure using larvae obtained from the same hives as those used to initiate the definitive exposure. Dimethoate was used as the reference toxicant and is known to be toxic to the honey bee larvae; therefore, the reference test was terminated at the end of the larval phase (day 8).

A 20 mg a.i./mL primary stock solution was prepared by bringing 2.0295 g of dimethoate (2.0254 g as active ingredient) to a volume of 100 mL with acetone (CAS No. 67-64-1). The resulting 20 mg a.i./mL reference toxicant solution was observed to be clear and colorless with no visible undissolved material following initial sonication of 20 seconds and mixing with a stir bar and magnetic stir plate for 10 minutes. A 12 mg a.i./mL solution was prepared by adding 6.0 mL of the 20 mg a.i./mL reference toxicant stock solution to a 10 mL volumetric flask and bringing it to volume with acetone. A single treated diet was prepared using the following preparation scheme:

Stock Concentration (mg a.i./mL)	Volume of Stock Solution Used (mL)	Total Weight of Diet (g)	Diet Concentration (µg a.i./g)	Total Amount Diet Dispensed per Well over Days 3, 4, 5, and 6[a]		Nominal Cumulative Dose (µg a.i./larva)
				(µL)	(g)	
12	0.040	10	48	140	0.160	7.9

[a] The weighted density is based on proportions of each diet type (B and C) within the total amount of diet dispensed per well (µL) as would be used in larval chronic testing. The weighted density is 1.1710 g/mL.

NOTE: Resulting diet concentration was calculated using the actual unrounded results and not the rounded values presented in this table.

The resulting reference toxicant diet was observed to be opaque and yellow in color with no visible undissolved material following preparation.

Experimental Initiation

The test was initiated on day 3, when the appropriate treated or control diet B was added to the larval cell plates.

Feeding during Exposure Phase

On day 3, individual larvae in all plates were fed 20 µL of the appropriate diet B; on days 4, 5, and 6, respectively, all plates were fed 30, 40, and 50 µL of the appropriate diet C. Larvae that were observed to completely consume their diet on day 7 or 8 were transferred to the appropriate, labeled pupation plates. On day 8, any larvae that did not consume the entire diet were considered dead.

Test Conditions

The larval exposure was conducted in the same incubator used for the acclimation phase, which was designed to maintain a temperature of 33 ± 2 °C with a relative humidity of ≥90%. The larvae, in their cell plates, were kept inside a plastic container in the incubator. The incubator maintained the test organisms in near darkness to approximate hive conditions. Organisms were exposed to laboratory lighting for approximately 30 minutes each day during observations and renewal of the diet.

Upon transfer to the pupal plates, the plates were maintained within an incubator at the same temperature but the relative humidity was designed to be maintained at 50 to 85%.

Temperature and relative humidity within a surrogate cell plate, placed in the incubator among the test plates, were monitored continuously using a HOBO data logger (Onset Computer Corporation, Model ZW-007).

Test Monitoring

The health of the larvae was observed and recorded daily. Death of a larva was defined by lack of movement. On days 7 and 8, all wells were observed for the presence of diet, and larvae that had not completely consumed their diet were considered dead. Survival of pupae was first checked on day 15 to avoid disturbing the fragile pre-pupal stage earlier. Larvae that failed to develop into pupae by day 15 were classified as dead. Starting on day 15, the number of emerged adults each day was recorded. At the time of emergence, each adult bee was removed from the well plate and individually weighed on a Sartorius Model SECURA225D-1S balance to the nearest 0.0001 g, and discarded.

Experimental Termination

The test was terminated on day 22. At test termination, after health observations and remaining individual bee weights were recorded, any remaining organisms were frozen and discarded. Pupae that had not emerged as adults by day 22 were considered dead.

Analytical Measurements

Analytical measurements were conducted by Pioneer Hi-Bred, International. Fifteen samples of test diet per treatment and five samples of control diet were collected on days 4, 5, and 6 of the exposure into tubes provided by Pioneer Hi-Bred, International. The 0.025 µg a.i./larva/day diet samples were collected from the beginning, middle and end of diet distribution; the 0.050 µg a.i./larva/day diet samples were collected from the end. In addition, all remaining aliquots of the stock solution were returned to Pioneer Hi-Bred, International. Results of these analyses

were used to characterize the stock solutions and confirm the treated diets were correctly prepared and to quantify actual exposure concentrations.

All samples were stored at -80 ± 10 °C prior to shipping. Samples were shipped on dry ice to Pioneer Hi-Bred, International and held frozen until analysis.

Endpoints and Statistical Analysis

The endpoints used for determination of significant effects by statistical evaluation are outlined below:

- Larval percent survival (number of live pupae on day 8 divided by the number of larvae exposed, 36 × 100)
- Pupal percent survival (number emerged adults on day 22 divided by the number live on day 8 × 100)
- Day 22 percent emergence (number of emerged adults divided by the number exposed, 36 × 100)
- Honey bee weight at emergence

Determination of LOED and NOED Values

The treatment data was tested for normality and homogeneity of variance using the appropriate qualifying test. The Lowest-Observed-Effect Dose (LOED) is defined as the lowest cumulative dose that shows a statistically significant reduction and the No-Observed-Effect Dose (NOED) is the highest dose that shows no statistically significant reduction from the control. Basic guidance for this determination is as follows but the actual statistical analysis utilized was dependent on the data set:

Endpoint	Normal Distribution[a]	Equal Variance[b]	Statistical Test
Larval Survival **Pupal Survival** **Emergence**	**Not Applicable**	**Not Applicable**	**Monotonic Trend:** Cochran-Armitage's Step-Down Test **Non-Monotonic Trend:** Fisher's Exact Test with Bonferroni-Holm's Adjustment
Weight	**Yes**	**Yes**	**Monotonic Trend:** Williams' Multiple Comparison Test or Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunnett's Multiple Comparison Test
	No	**Yes or No**	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunn's Test or Wilcoxon's Test with Bonferroni-Holm's Adjustment
	Yes	**No**	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Tamhane-Dunnett Test or Dunnett's T3 Test

[a] Shapiro-Wilks' Test was used to assess normality of variance.
[b] Bartlett's Test was used to assess homoscedasticity.

All comparisons for determination of a NOED and LOED were made at ≥95% level of certainty ($p < 0.05$) and compared on a per replicate basis. A replicate was considered to be an individual larva/bee since they were reared in an individual cell. CETIS Version 1.8 was used to perform all statistical analysis. Results are reported in µg a.i./larva for cumulative dose (NOED and LOED values).

RESULTS

Definitive Testing

Environmental Conditions

Larval Conditions

The daily minimum and maximum temperatures monitored in the cell plates ranged from 33 to 34 °C and relative humidity ranged from 89 to 97% during the larval phase (e.g., days 1 to 8). Based on historical data, these parameters were considered acceptable for the survival and growth of the test organisms.

Pupal Conditions

The temperature ranged from 32 to 35 °C with a relative humidity range of 60 to 87% during the pupal phase (e.g., days 7 to 22). Based on historical data, these parameters were considered acceptable for the survival and growth of the test organisms.

Biological Results

IPD072Aa Protein Exposure

Table 186, Table 187, and Table 188present the results of biological exposure. Table 189 provides a summary of percent survival, adult emergence, and adult weight at emergence. The larval survival (days 3 to 8) was 92, 86, and 92% honey bee larvae in the control, 0.10, and 0.20 µg a.i./larva treatments, respectively.

Fisher's Exact Test with Bonferroni-Holm's Adjustment determined no significant reduction in larval survival among honey bees exposed to the 0.10 and 0.20 µg a.i./larva treatment levels compared to the control. Therefore, the 8-day larval survival NOED and LOED values for IPD072Aa protein to honey bees were determined to be 0.20 and >0.20 µg a.i./larva, respectively.

The pupal survival (days 8 to 22) was 82, 94, and 94% in the control, 0.10, and 0.20 µg a.i./larva treatments, respectively.

Fisher's Exact Test with Bonferroni-Holm's Adjustment determined no significant reduction in pupal survival among honey bees exposed to the 0.10 and 0.20 µg a.i./larva treatment levels compared to the control. Therefore, the 22-day pupal survival NOED and LOED values for IPD072Aa protein to honey bees were determined to be 0.20 and >0.20 µg a.i./larva, respectively.

At test termination, the adult percent emergence (days 3 to 22) in the control, 0.10, and 0.20 µg a.i./larva treatments was 75, 81, and 86%, respectively.

Fisher's Exact Test with Bonferroni-Holm's Adjustment determined no significant reduction in adult percent emergence for honey bees exposed to the 0.10 and 0.20 µg a.i./larva treatment levels compared to the control. Therefore, the 22-day adult emergence NOED and LOED values for IPD072Aa protein to honey bees were determined to be 0.20 and >0.20 µg a.i./larva/day, respectively.

Mean live weight for adults at emergence is presented in Table 188. The mean adult weight at emergence in the control, 0.10, and 0.20 µg a.i./larva treatments was 0.1081, 0.1095, and 0.1068 g, respectively.

Dunn's Test with Bonferroni-Holm's Adjustment determined no significant reduction in live weight for adults at emergence among honey bees exposed to the 0.10 and 0.20 µg a.i./larva treatment levels, compared to the control. Therefore, the NOED and LOED values were determined to be 0.20 and >0.20 µg a.i./larva, respectively.

Dimethoate Exposure (Reference Test)

Table 186 presents the results of biological exposure. The nominal cumulative dose rate of dimethoate maintained during the reference test was 7.9 µg a.i./larva equivalent to 48 µg a.i./g diet. Procedures used during the reference test were consistent with the procedures used in the definitive test. Mortality during the larval stage (days 3 to 8) was 97% for honey bee larvae exposed to 7.9 µg a.i./larva nominal cumulative dose. These results demonstrate that the larvae were sensitive to dimethoate in this exposure system.

ACCEPTABILITY CRITERIA

The following acceptance criteria were required:

Acceptability Criteria	Study Results	Criterion Met (Yes/No)
Larval mortality from days 3 to 8 in the control, and solvent control, if present, should be ≤15% prior to pupation.	Larval mortality in the control was 8%.	Yes
Percent emergence in the control, and solvent control, if present, should be ≥70% at termination.	Emergence in the control was 75%.	Yes
Larval mortality in the reference toxicant treatment level (7.9 µg a.i. dimethoate/larva) should be ≥50% on day 8.	Larval mortality in the 7.9 µg a.i. dimethoate/larva treatment was 97%.	Yes

CONCLUSIONS

The 8-day larval, 22-day pupal, and 22-day percent emergence NOED and LOED values for IPD072Aa protein to honey bees were all determined to be 0.20 and >0.20 µg a.i./larva, respectively. The live weight for adults at emergence NOED and LOED values for IPD072Aa protein values were also determined to be 0.20 and >0.20 µg a.i./larva, respectively.

Table 190 summarizes the established endpoints for this study (LOED and NOED values) based on the nominal cumulative dose. Results of the toxic reference standard test (dimethoate) indicated that the test organisms were responsive to a toxicant of the appropriate dose in this study design.

Table 186. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to IPD072Aa Protein - Larval Survival and Mortality

Nominal Cumulative Dose (µg a.i./larva)	Percent Survival (Number of Surviving Larvae)					Day 8		
	Day 3	Day 4	Day 5	Day 6	Day 7	Percent Survival (Number of Surviving Larvae)	Percent Mortality (Number of Dead Larvae)	Abbott's Corrected Percent Mortality[a]
Control	100 (36)	100 (36)	100 (36)	100 (36)	100 (36)	92 (33)	8 (3)	NA[b]
0.10	100 (36)	100 (36)	97 (35)	94 (34)	92 (33)	86 (31)	14 (5)	6
0.20	100 (36)	100 (36)	97 (35)	97 (35)	97 (35)	92 (33)	8 (3)	0

[a] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925). Statistical analysis was conducted using uncorrected values.
[b] NA = Not Applicable

Dimethoate Reference Test

Nominal Cumulative Dose (µg a.i./larva)	Cumulative Percent Survival (Number of Surviving Larvae)					Day 8	
	Day 3	Day 4	Day 5	Day 6	Day 7	Percent Survival (Number of Surviving Larvae)	Percent Mortality (Number of Dead Larvae)
7.9	100 (36)	100 (36)	47 (17)	25 (9)	25 (9)	3 (1)	97 (35)

NOTE: A total of 36 organisms per treatment or control at exposure initiation, day 3.

Table 187. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to IPD072Aa Protein - Pupal Survival and Adult Percent Emergence

Nominal Cumulative Dose (µg a.i./larva)	N [a]	Day 8 - 22			Day 3 - 22		
		Cumulative Percent Survival (Number of Surviving Pupae)	Cumulative Percent Mortality (Number of Dead Pupae)	Abbott's Corrected Percent Mortality[b]	Cumulative Percent Emergence[c] (Number of Emerged Adults)	Cumulative Percent Mortality[c] (Number of Dead Organisms)	Abbott's Corrected Percent Mortality[b]
Control	3 3	82 (27)	18 (6)	NA[d]	75 (27)	25 (9)	NA
0.10	3 1	94 (29)	6 (2)	-14	81 (29)	19 (7)	-7
0.20	3 3	94 (31)	6 (2)	-15	86 (31)	14 (5)	-15

[a] N = the number of larvae transferred
[b] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925). Statistical analysis was conducted using uncorrected values.
[c] Based on 36 larvae at initiation
[d] NA = Not Applicable

Table 188. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to IPD072Aa Protein - Adult Weight at Emergence

Nominal Cumulative Dose (µg a.i./larva)	N[a]	Mean Adult Weight at Emergence[b] (g)
Control	27	0.1081 (0.0146)
0.10	29	0.1095 (0.0101)
0.20	31	0.1068 (0.0229)

[a] N = the number of adults weighed
[b] Standard deviations are presented in parentheses.

Table 189. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to IPD072Aa Protein - Summary of the Percent Survival, Adult Emergence, and Adult Weight at Emergence

Nominal Cumulative Dose (µg a.i./larva)	8-Day Larval Survival[a] (%)	22-Day Pupal Survival[b] (%)	22-Day Percent Emergence[c] (%)	Adult Weight at Emergence (g)
Control	92	82	75	0.1081
0.10	86	94	81	0.1095
0.20	92	94	86	0.1068

[a] Based on observations from days 3 to 8
[b] Based on observations from days 8 to 22
[c] Based on observations from days 3 to 22

Table 190. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to IPD072Aa Protein - Endpoint Summary

Endpoint	Based on Nominal Cumulative Dose (µg a.i./larva)	
	NOED[a]	LOED[b]
3 - 8-Day Larval Survival	0.20	>0.20
8 - 22-Day Pupal Survival	0.20	>0.20
3 - 22-Day Adult Emergence	0.20	>0.20
Adult Weight at Emergence	0.20	>0.20

[a] NOED = No-Observed-Effect Dose
[b] LOED = Lowest-Observed-Effect Dose

Analytical Results

Characterization of Larval and Adult Honey Bee Diets Incorporated with IPD072Aa Protein

The objective of this study was to prepare a working stock solution of IPD072Aa protein test substance for incorporation into larval and adult honey bee diets and to characterize those diets.

IPD072Aa protein test substance was diluted in ultrapure water to a concentration of 550 ng/µl to create a working stock solution. Aliquots of the working stock solution were shipped to the CRO and used to prepare larval and adult honey bee diets under study numbers PHI-2018-053

and PHI-2018-052, respectively. The prepared diet samples from the honey bee bioassays were shipped to Pioneer Hi-Bred International, Inc. for characterization.

Western blot analysis was used to visually confirm the concentration and homogeneity of IPD072Aa protein in larval test diets, concentration of IPD072Aa protein in adult test diets, and absence of IPD072Aa protein in larval and adult control diets. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in the working stock solution following frozen storage.

An IPD072Aa protein working stock solution was provided to the CRO for use in larval and adult honey bee diets and those diets were characterized as applicable.

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Test System

The test system is not applicable for this study.

Experimental Design

IPD072Aa protein test substance was diluted in ultrapure water to a concentration of 550 ng/µl to create a working stock solution. Aliquots of the working stock solution were shipped to the CRO and used to prepare larval and adult honey bee diets. In addition, extra aliquots of working stock solution were shipped to the CRO and then returned once the honey bee bioassays were complete. These aliquots were used in the sensitive insect bioassay to confirm the biological activity of IPD072Aa protein. Prepared diet samples from the honey bee bioassays were shipped to Pioneer Hi-Bred International, Inc. (referred to as Pioneer) for characterization.

Western blot analysis was used to visually confirm the concentration and homogeneity of IPD072Aa protein in larval test diets, the concentration of IPD072Aa protein in adult test diets, and the absence of IPD072Aa protein in larval and adult control diets. Homogeneity verification of the adult honey bee diet was not applicable because the diet was a solution. A sensitive insect bioassay with WCR (see section G2.a. Honey Bee IPD072Aa Protein Sensitive Insect

Bioassay) was conducted to demonstrate the biological activity of the IPD072Aa protein in the stored working stock solution.

Control of bias during characterization was achieved through the use of replicate testing. In addition, the working stock solution was prepared in bulk, minimizing day to day bias. Bias in the WCR bioassay was controlled through the randomization of treatments within blocks.

Preparation and Shipping of Working Stock Solution

IPD072Aa protein test substance was removed from frozen storage and allowed to thaw in a 4 ºC refrigerator or on wet ice. The test substance was diluted in chilled ultrapure water to create an IPD072Aa protein working stock solution to a concentration of 550 ng/µl. Prior to shipment, the working stock solution was aliquoted, flash frozen with liquid nitrogen, and stored frozen (-80 °C freezer unit).

Aliquots of the IPD072Aa protein working stock solution were shipped to the CRO on dry ice for use in preparing diets for larval and adult honey bee bioassays. Additional aliquots of the working stock solution were returned to Pioneer on dry ice after completion of the honey bee bioassays for use in preparing test diet for the sensitive insect bioassay.

Diet Sample Collection

Diets were prepared at the CRO for use in larval and adult honey bee bioassays. During the process of diet distribution, samples were collected as follows:

On days 4, 5, and 6 of the larval honey bee bioassay, 15 samples per test diet (25.0 and 50.0 ng active ingredient (a.i.)/larva/day; 150 µl each) were collected during diet distribution for homogeneity verification and/or verification of concentration. In addition, 5 samples of control diet (150 µl each) were collected on each of those days to verify the absence of IPD072Aa protein.

On days 2, 6, and 9 of the adult honey bee bioassay, 5 samples per test diet (0.5 and 1.0 µg a.i./bee/day; 100 µl each) and 5 samples of control diet (100 µl each) were collected for verification of the concentration or absence of IPD072Aa protein in adult test and control diets, respectively.

Characterization of Working Stock Solution and Diets

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples (60 µl) of collected larval diets were extracted in 600 µl of 1X lithium dodecyl sulfate (LDS) sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing dithiothreitol (DTT), and 65% water) in preparation for SDS-PAGE. In addition, samples of the collected adult diets were allowed to thaw and prepared for SDS-PAGE by diluting as applicable in 1X LDS/DTT. A sub-sample of the test substance was prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. For larval diet dose confirmation, additional sub-samples of the SDS-PAGE-prepared test substance were diluted in control diet matrix. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown inTable 191.

Table 191. Larval and Adult Honey Bee IPD072Aa Diet Samples

Bioassay	Assessment	Diet	Days	Number of Samples
Larval Honey Bee	Homogeneity	25.0 ng a.i./Larva/Day	4, 5, 6	9[a] per Timepoint
		50.0 ng a.i./Larva/Day	4, 5, 6	9[b] per Timepoint
Larval Honey Bee	Dose Confirmation of IPD072Aa Protein	25.0 ng a.i./Larva/Day	4, 5, 6	3 per Timepoint
		50.0 ng a.i./ Larva/Day	4, 5, 6	3 per Timepoint
Adult Honey Bee		0.5 µg a.i./Bee/Day	2, 6, 9	3 per Timepoint
		1.0 µg a.i./Bee/Day	2, 6, 9	3 per Timepoint
Larval Honey Bee	Absence of IPD072Aa Protein	Control	4, 5, 6	3 per Timepoint
Adult Honey Bee		Control	2, 6, 9	3 per Timepoint

[a] Three samples each from the beginning, middle, and end of diet distribution.
[b] Nine samples from the end of diet distribution.

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). For larval diet dose confirmation, sub-samples of the test substance diluted in control diet matrix were also loaded to the gels. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:10,000 or 1:20,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 or 1:20,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix.

Demonstration of IPD072Aa Protein Activity in Working Stock Solution

Aliquots of the stored IPD072Aa protein working stock solution were returned to Pioneer by the CRO on dry ice after completion of the honey bee bioassays. Returned aliquots were used to prepare Treatment B in a sensitive insect bioassay using *WCR* larvae to demonstrate the biological activity of IPD072Aa protein in the working stock solution used to make the honey bee diets. Details regarding the sensitive insect bioassay are provided in section G2.a. Honey Bee IPD072Aa Protein Sensitive Insect Bioassay.

Results and Discussion

An IPD072Aa working stock solution was prepared by Pioneer and used by the CRO to prepare larval and adult honey bee diets. Samples of the prepared diets were shipped to Pioneer for characterization by western blot analysis.

Western blot analysis visually confirmed the homogeneity of IPD072Aa protein in larval test diets (Figure 76; Figure 77; Figure 79; Figure 80; Figure 82; Figure 83). The concentration of IPD072Aa protein in larval test diets (Figure 78; Figure 81; Figure 84) was also visually confirmed, with the exception of the 25 ng a.i. larval diet on Day 6, which appeared lower than the targeted IPD072Aa protein concentration. However, as this dose was visually confirmed on Days 4 and 5 and the concentration of the higher dose (50 ng a.i.) was visually confirmed for days 4, 5, and 6, it is reasonable to assume the honey bees were exposed to at least the 50 ng a.i. concentration.

Western blot analysis visually confirmed the concentration of IPD072Aa protein in adult test diets (Figure 85; Figure 86; Figure 87). The absence of IPD072Aa protein in the larval and adult honey bee control diets was also verified. No IPD072Aa protein band was detected in any of the control diets (Figure 76; Figure 77; Figure 78; Figure 79; Figure 80; Figure 81; Figure 82; Figure 83; Figure 84for larval diets; Figure 85; Figure 86; Figure 87 for adult diets).

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 192. The WCR bioassay met the acceptability criterion (section G2.a. Honey Bee IPD072Aa Protein Sensitive Insect Bioassay). The biological activity of the IPD072Aa protein in returned aliquots of the stored working stock solution used in the honey bee bioassays was demonstrated by 100% mortality of WCR fed the test diet (Treatment B; described in the Sensitive Insect Bioassay section) when compared to the bioassay control diet (Treatment A).

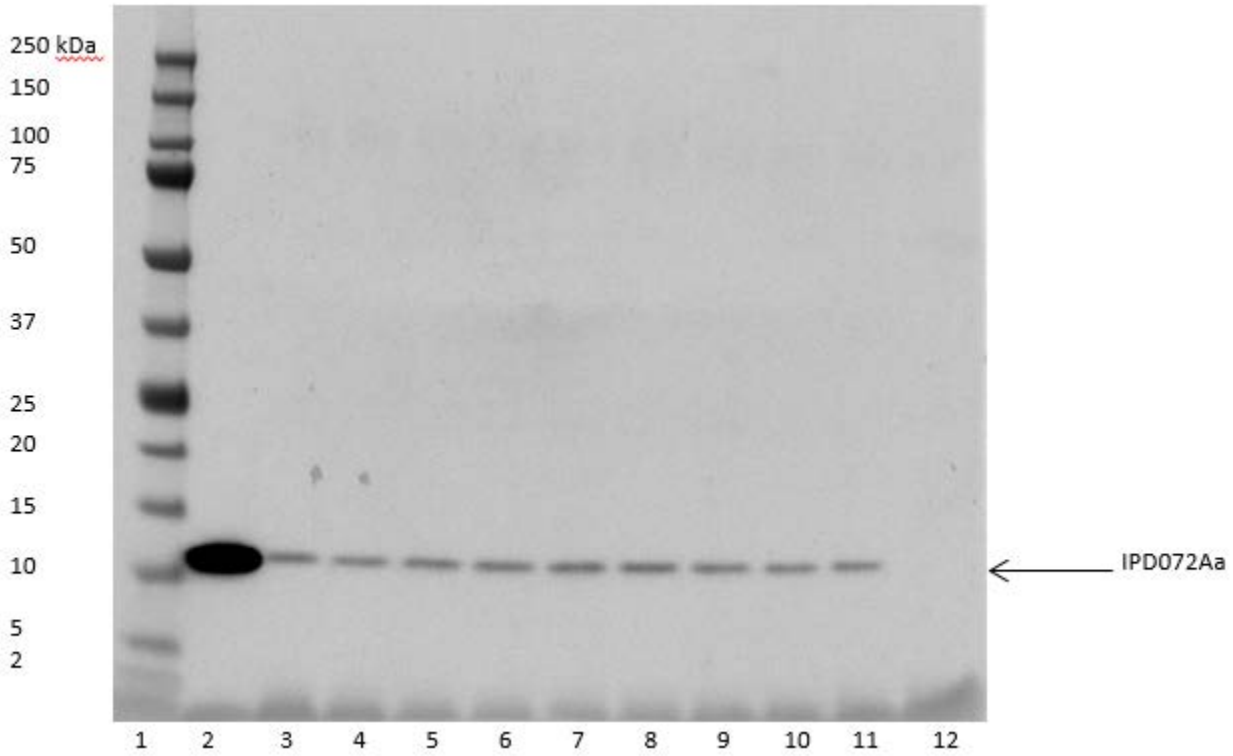
Conclusion

An IPD072Aa protein working stock solution was provided to the CRO for use in larval and adult honey bee diets and those diets were characterized as applicable.

Table 192. Summary of IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0	30	2	6.67
B	Test Diet	393.25	30	30	100

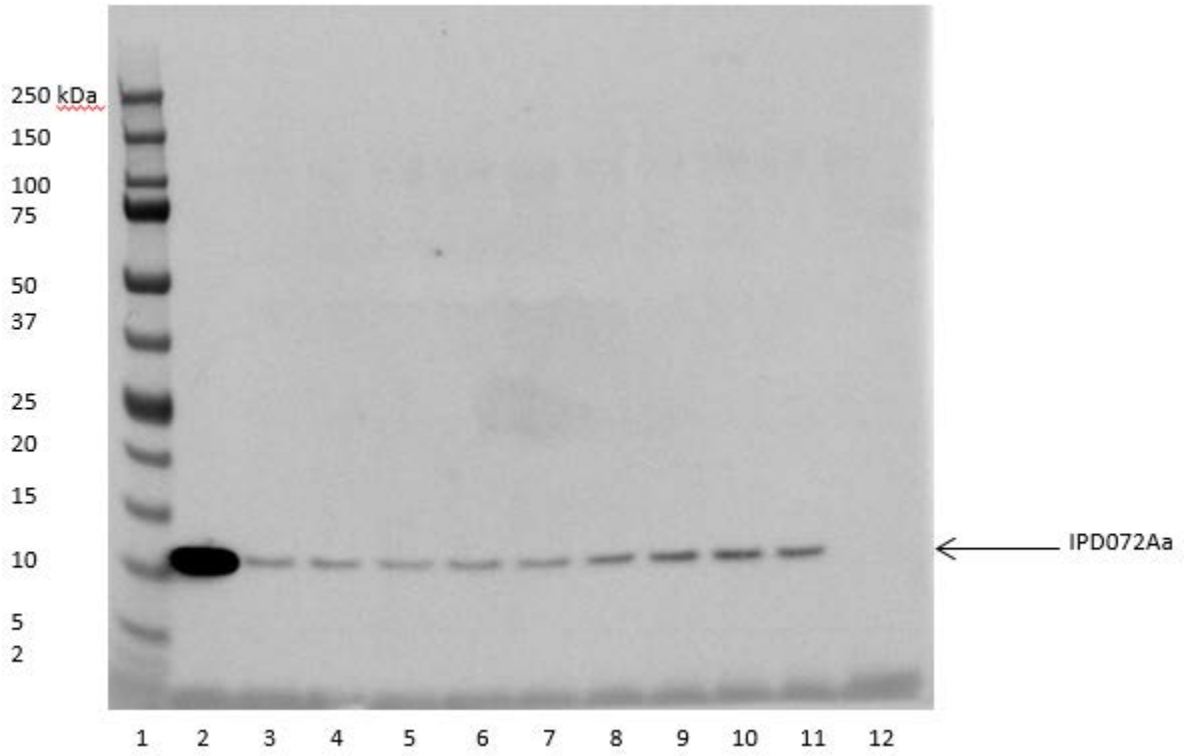
Note: Treatment B was prepared from the same working stock solution used to prepare diets for the larval and adult honey bee bioassays following storage. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	25 ng a.i. IPD072Aa Protein Diet (Beginning)
4	25 ng a.i. IPD072Aa Protein Diet (Beginning)
5	25 ng a.i. IPD072Aa Protein Diet (Beginning)
6	25 ng a.i. IPD072Aa Protein Diet (Middle)
7	25 ng a.i. IPD072Aa Protein Diet (Middle)
8	25 ng a.i. IPD072Aa Protein Diet (Middle)
9	25 ng a.i. IPD072Aa Protein Diet (End)
10	25 ng a.i. IPD072Aa Protein Diet (End)
11	25 ng a.i. IPD072Aa Protein Diet (End)
12	Control Diet Sample

Note: Nanogram (ng) and active ingredient (a.i.). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. The control diet sample was loaded at the same dilution as the IPD072Aa protein homogeneity samples. Homogeneity samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in the diet.

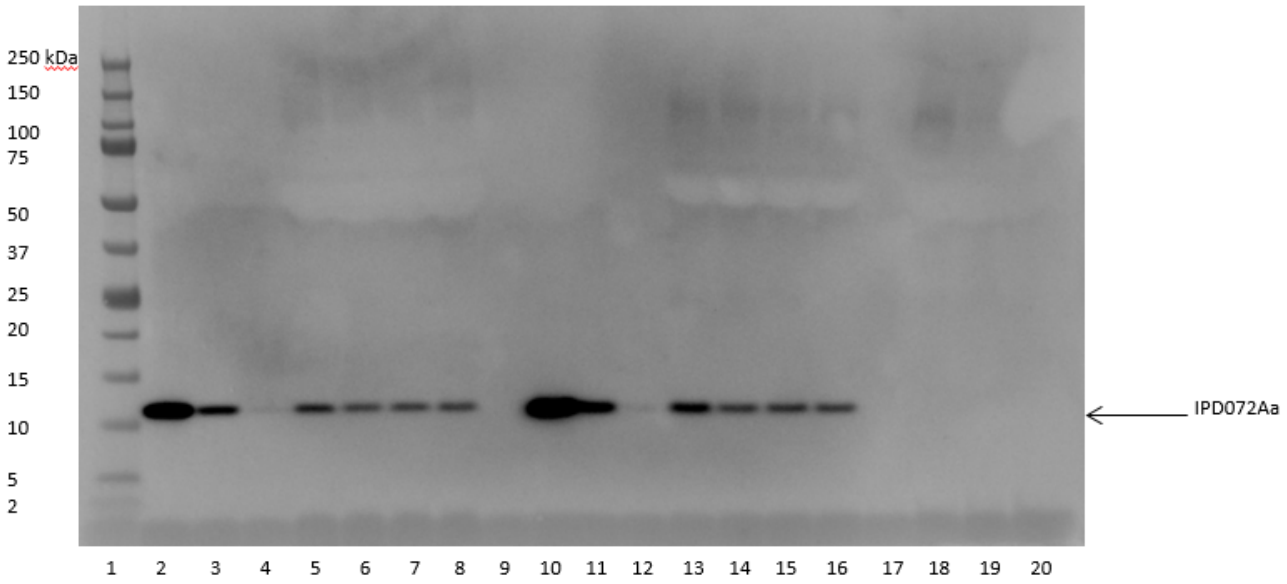
Figure 76. Homogeneity Assessment of the IPD072Aa Protein in Larval Diet (25 ng a.i.), Day 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	50 ng a.i. IPD072Aa Protein Diet (Rep 1)
4	50 ng a.i. IPD072Aa Protein Diet (Rep 2)
5	50 ng a.i. IPD072Aa Protein Diet (Rep 3)
6	50 ng a.i. IPD072Aa Protein Diet (Rep 4)
7	50 ng a.i. IPD072Aa Protein Diet (Rep 5)
8	50 ng a.i. IPD072Aa Protein Diet (Rep 6)
9	50 ng a.i. IPD072Aa Protein Diet (Rep 7)
10	50 ng a.i. IPD072Aa Protein Diet (Rep 8)
11	50 ng a.i. IPD072Aa Protein Diet (Rep 9)
12	Control Sample

Note: Nanogram (ng) and active ingredient (a.i.). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. The control sample was loaded at the same dilution as the IPD072Aa protein homogeneity samples. Homogeneity samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in the diet.

Figure 77. Homogeneity Assessment of the IPD072Aa Protein in Larval Diet (50 ng a.i.), Day 4



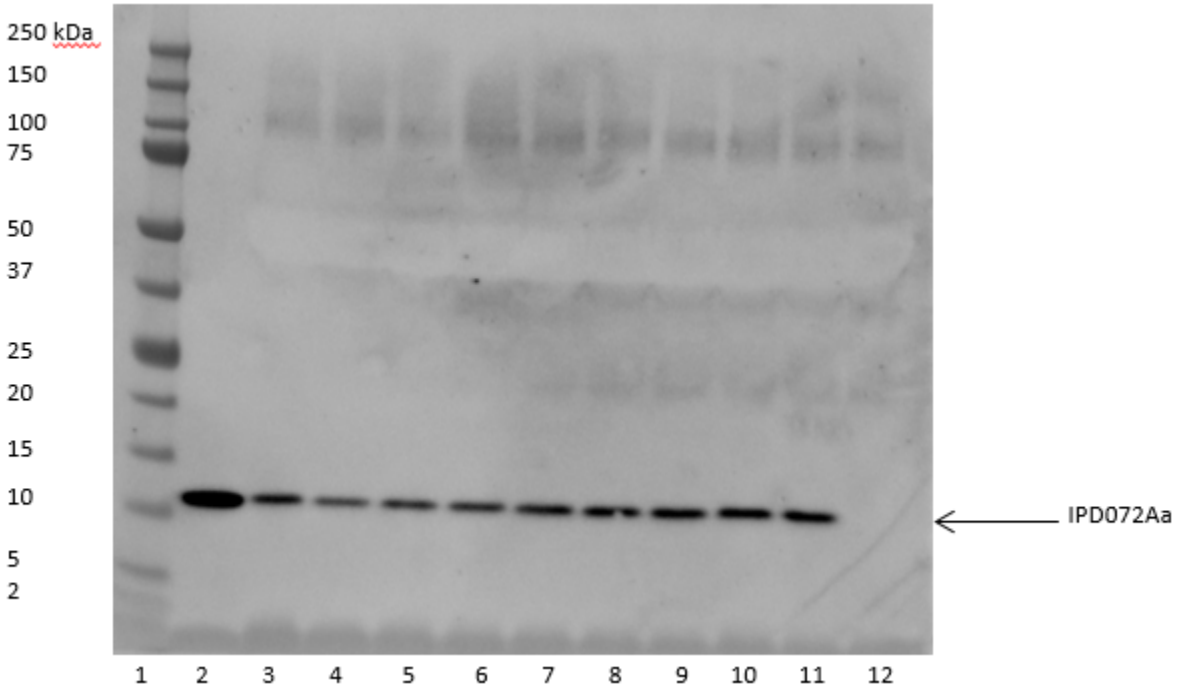
Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Test Substance (1 ng)
2	Test Substance (5 ng)	12	Test Substance (0.2 ng)
3	Test Substance (1 ng)	13	Test Substance (1 ng)[b]
4	Test Substance (0.2 ng)	14	50 ng a.i. IPD072Aa Protein Diet
5	Test Substance (1 ng)[a]	15	50 ng a.i. IPD072Aa Protein Diet
6	25 ng a.i. IPD072Aa Protein Diet	16	50 ng a.i. IPD072Aa Protein Diet
7	25 ng a.i. IPD072Aa Protein Diet	17	1X LDS/DTT Sample Buffer Blank
8	25 ng a.i. IPD072Aa Protein Diet	18	Control Diet Sample
9	1X LDS/DTT Sample Buffer Blank	19	Control Diet Sample
10	Test Substance (5 ng)	20	Control Diet Sample

Note: Nanogram (ng), active ingredient (a.i.), and lithium dodecyl sulfate with dithiothreitol (LDS/DTT). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Control samples were loaded at the same dilution as the 25 ng a.i. dose confirmation diet samples. Dose confirmation samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in each respective diet.

[a] Test substance was diluted in control diet matrix equivalent to the 25 ng a.i. diet.

[b] Test substance was diluted in control diet matrix equivalent to the 50 ng a.i. diet.

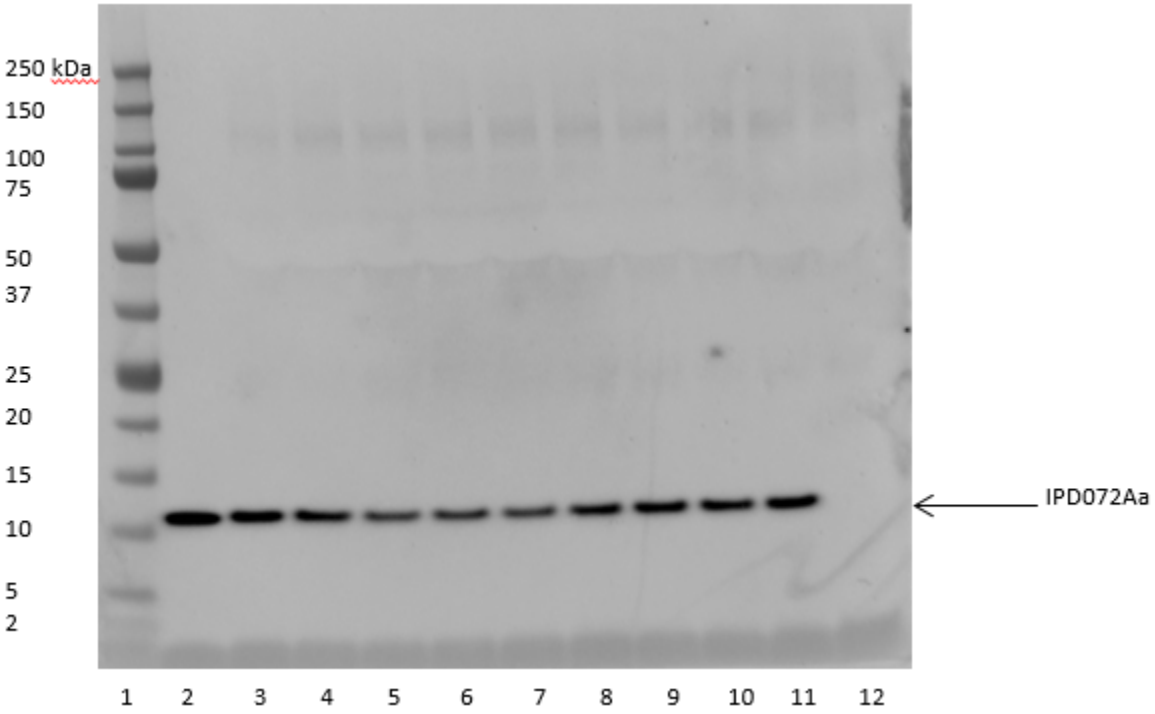
Figure 78. Dose Confirmation of the IPD072Aa Protein in Larval Diets (25 and 50 ng a.i.) and Assessment of the Absence of IPD072Aa Protein in Control Diet, Day 4



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (1 ng)
3	25 ng a.i. IPD072Aa Protein Diet (Beginning)
4	25 ng a.i. IPD072Aa Protein Diet (Beginning)
5	25 ng a.i. IPD072Aa Protein Diet (Beginning)
6	25 ng a.i. IPD072Aa Protein Diet (Middle)
7	25 ng a.i. IPD072Aa Protein Diet (Middle)
8	25 ng a.i. IPD072Aa Protein Diet (Middle)
9	25 ng a.i. IPD072Aa Protein Diet (End)
10	25 ng a.i. IPD072Aa Protein Diet (End)
11	25 ng a.i. IPD072Aa Protein Diet (End)
12	Control Sample

Note: Nanogram (ng) and active ingredient (a.i.). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. The control sample was loaded at the same dilution as the IPD072Aa protein homogeneity samples. Homogeneity samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in the diet.

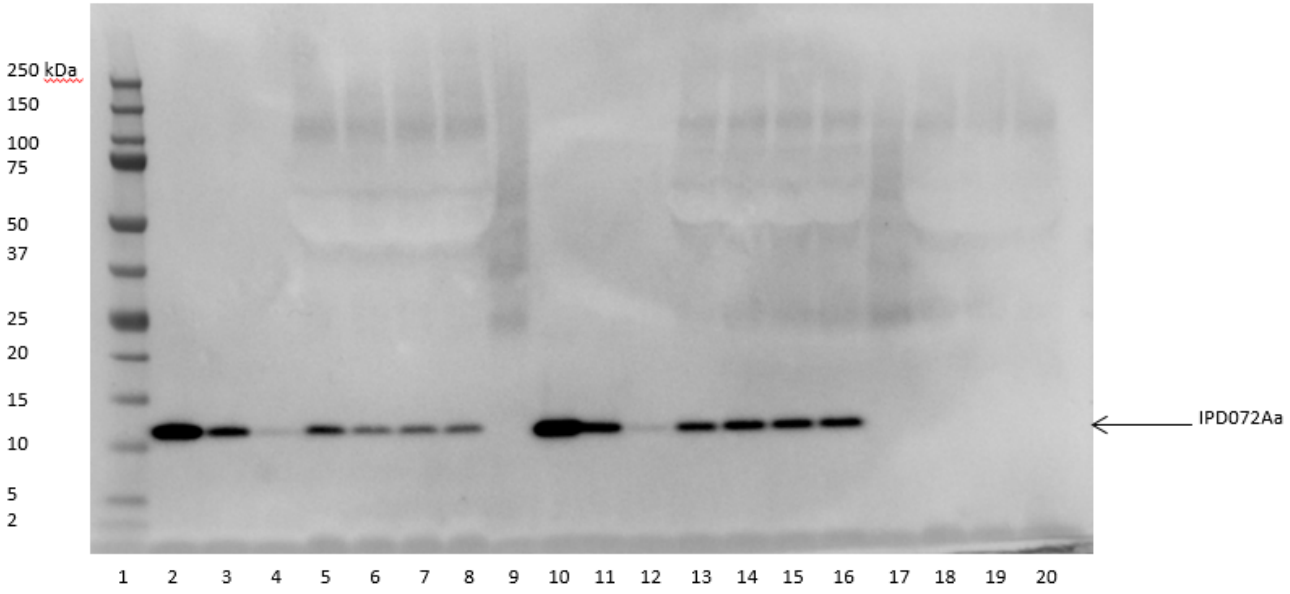
Figure 79. Homogeneity Assessment of the IPD072Aa Protein in Larval Diet (25 ng a.i.), Day 5



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (1 ng)
3	50 ng a.i. IPD072Aa Protein Diet (Rep 1)
4	50 ng a.i. IPD072Aa Protein Diet (Rep 2)
5	50 ng a.i. IPD072Aa Protein Diet (Rep 3)
6	50 ng a.i. IPD072Aa Protein Diet (Rep 4)
7	50 ng a.i. IPD072Aa Protein Diet (Rep 5)
8	50 ng a.i. IPD072Aa Protein Diet (Rep 6)
9	50 ng a.i. IPD072Aa Protein Diet (Rep 7)
10	50 ng a.i. IPD072Aa Protein Diet (Rep 8)
11	50 ng a.i. IPD072Aa Protein Diet (Rep 9)
12	Control Sample

Note: Nanogram (ng) and active ingredient (a.i.). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. The control sample was loaded at the same dilution as the IPD072Aa protein homogeneity samples. Homogeneity samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in the diet.

Figure 80. Homogeneity Assessment of the IPD072Aa Protein in Larval Diet (50 ng a.i), Day 5



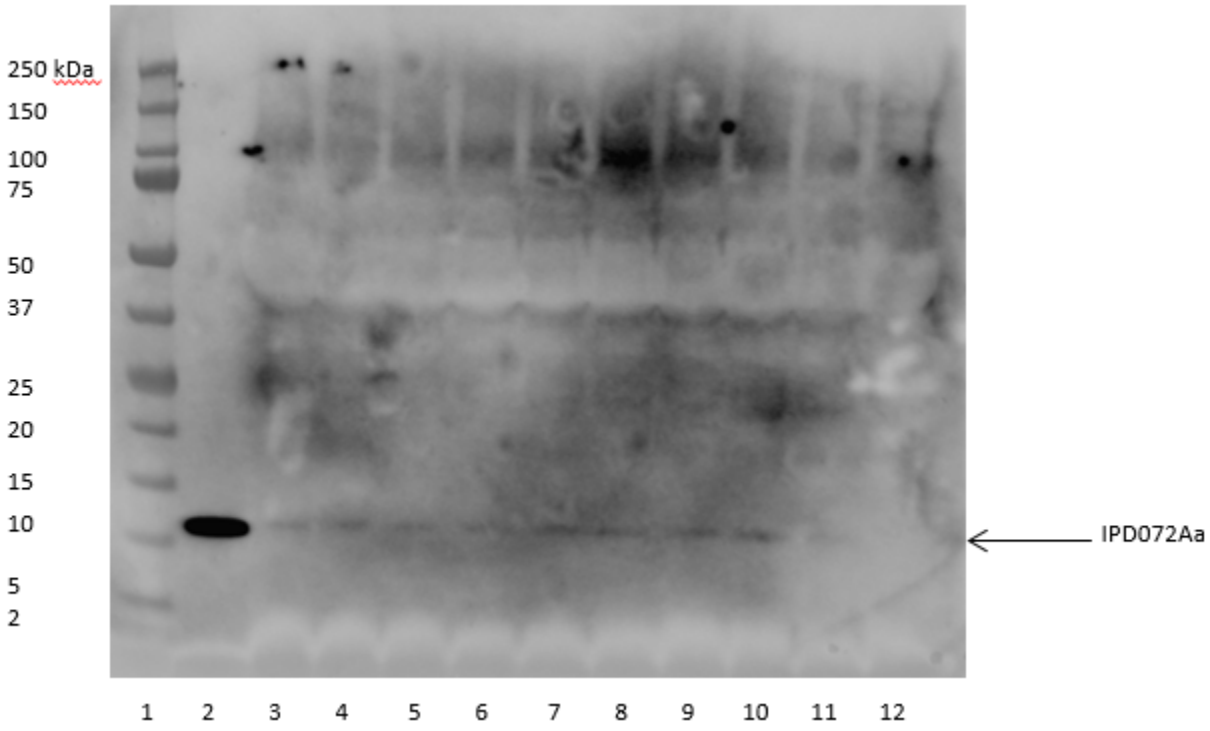
Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Test Substance (1 ng)
2	Test Substance (5 ng)	12	Test Substance (0.2 ng)
3	Test Substance (1 ng)	13	Test Substance (1 ng)[b]
4	Test Substance (0.2 ng)	14	50 ng a.i. IPD072Aa Protein Diet
5	Test Substance (1 ng)[a]	15	50 ng a.i. IPD072Aa Protein Diet
6	25 ng a.i. IPD072Aa Protein Diet	16	50 ng a.i. IPD072Aa Protein Diet
7	25 ng a.i. IPD072Aa Protein Diet	17	1X LDS/DTT Sample Buffer Blank
8	25 ng a.i. IPD072Aa Protein Diet	18	Control Diet Sample
9	1X LDS/DTT Sample Buffer Blank	19	Control Diet Sample
10	Test Substance (5 ng)	20	Control Diet Sample

Note: Nanogram (ng), active ingredient (a.i.), and lithium dodecyl sulfate with dithiothreitol (LDS/DTT). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Control samples were loaded at the same dilution as the 25 ng a.i. dose confirmation diet samples. Dose confirmation samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in each respective diet.

[a] Test substance was diluted in control diet matrix equivalent to the 25 ng diet.

[b] Test substance was diluted in control diet matrix equivalent to the 50 ng diet.

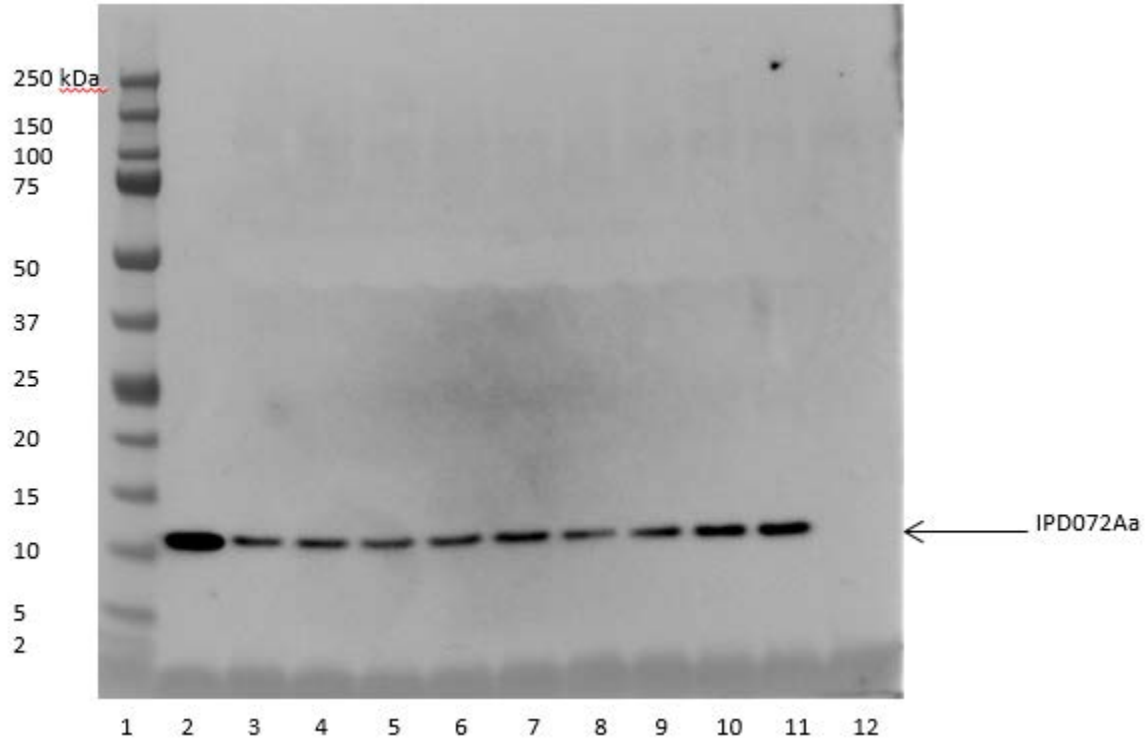
Figure 81. Dose Confirmation of the IPD072Aa Protein in Larval Diets (25 and 50 ng a.i.) and Assessment of the Absence of IPD072Aa Protein in Control Diet, Day 5



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (1 ng)
3	25 ng a.i. IPD072Aa Protein Diet (Beginning)
4	25 ng a.i. IPD072Aa Protein Diet (Beginning)
5	25 ng a.i. IPD072Aa Protein Diet (Beginning)
6	25 ng a.i. IPD072Aa Protein Diet (Middle)
7	25 ng a.i. IPD072Aa Protein Diet (Middle)
8	25 ng a.i. IPD072Aa Protein Diet (Middle)
9	25 ng a.i. IPD072Aa Protein Diet (End)
10	25 ng a.i. IPD072Aa Protein Diet (End)
11	25 ng a.i. IPD072Aa Protein Diet (End)
12	Control Sample

Note: Nanogram (ng) and active ingredient (a.i.). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. All samples were loaded undiluted. Homogeneity samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in the diet.

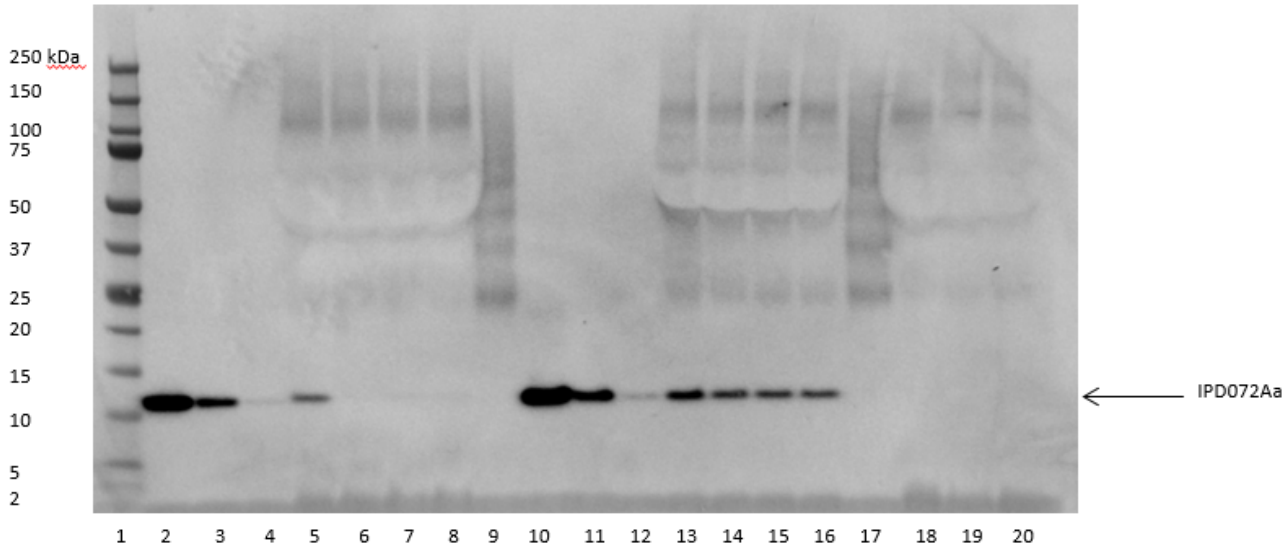
Figure 82. Homogeneity Assessment of the IPD072Aa Protein in Larval Diet (25 ng a.i.), Day 6



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (1 ng)
3	50 ng a.i. IPD072Aa Protein Diet (Rep 1)
4	50 ng a.i. IPD072Aa Protein Diet (Rep 2)
5	50 ng a.i. IPD072Aa Protein Diet (Rep 3)
6	50 ng a.i. IPD072Aa Protein Diet (Rep 4)
7	50 ng a.i. IPD072Aa Protein Diet (Rep 5)
8	50 ng a.i. IPD072Aa Protein Diet (Rep 6)
9	50 ng a.i. IPD072Aa Protein Diet (Rep 7)
10	50 ng a.i. IPD072Aa Protein Diet (Rep 8)
11	50 ng a.i. IPD072Aa Protein Diet (Rep 9)
12	Control Sample

Note: Nanogram (ng) and active ingredient (a.i.). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. The control sample was loaded at the same dilution as the IPD072Aa protein homogeneity samples. Homogeneity samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in the diet.

Figure 83. Homogeneity Assessment of the IPD072Aa Protein in Larval Diet (50 ng a.i.), Day 6



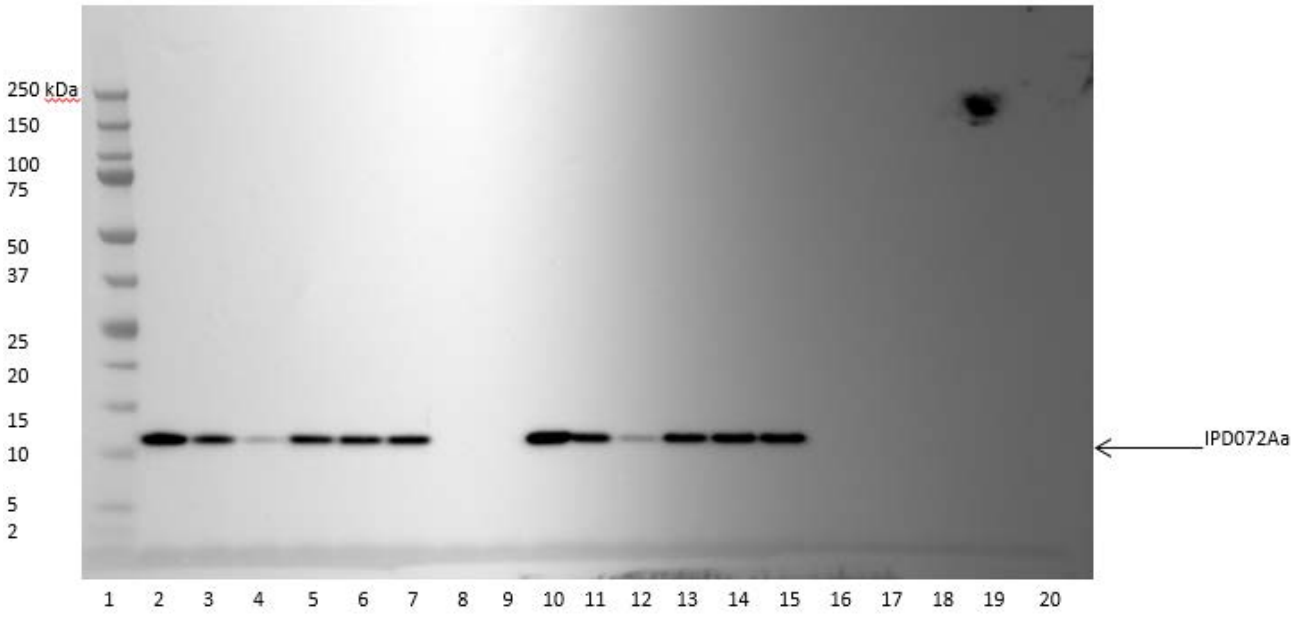
Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Test Substance (1 ng)
2	Test Substance (5 ng)	12	Test Substance (0.2 ng)
3	Test Substance (1 ng)	13	Test Substance (1 ng)[b]
4	Test Substance (0.2 ng)	14	50 ng a.i. IPD072Aa Protein Diet
5	Test Substance (1 ng)[a]	15	50 ng a.i. IPD072Aa Protein Diet
6	25 ng a.i. IPD072Aa Protein Diet	16	50 ng a.i. IPD072Aa Protein Diet
7	25 ng a.i. IPD072Aa Protein Diet	17	1X LDS/DTT Sample Buffer Blank
8	25 ng a.i. IPD072Aa Protein Diet	18	Control Diet Sample
9	1X LDS/DTT Sample Buffer Blank	19	Control Diet Sample
10	Test Substance (5 ng)	20	Control Diet Sample

Note: Nanogram (ng), active ingredient (a.i.), and lithium dodecyl sulfate with dithiothreitol (LDS/DTT). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Control samples were loaded undiluted. Dose confirmation samples were loaded to a target of 1 ng based on the nominal concentration of IPD072Aa protein in each respective diet.

[a] Test substance was diluted in control diet matrix equivalent to the 25 ng diet.

[b] Test substance was diluted in control diet matrix equivalent to the 50 ng diet.

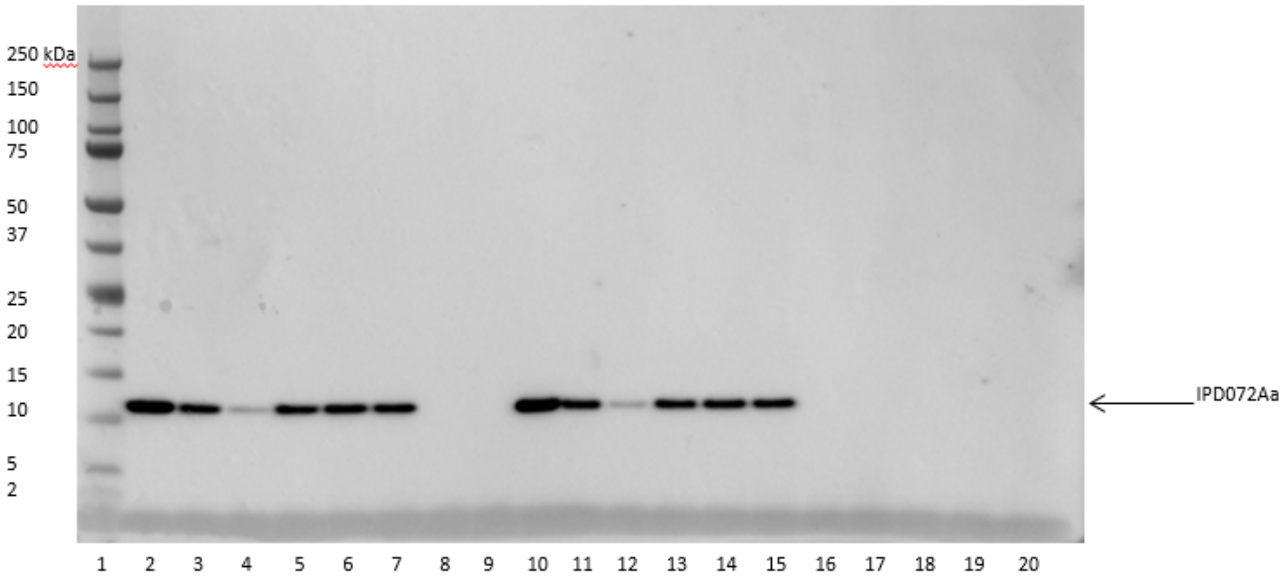
Figure 84. Dose Confirmation of the IPD072Aa Protein in Larval Diets (25 and 50 ng a.i.) and Assessment of the Absence of IPD072Aa Protein in Control Diet, Day 6



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Test Substance (5 ng)
2	Test Substance (10 ng)	12	Test Substance (1 ng)
3	Test Substance (5 ng)	13	1.0 µg a.i. IPD072Aa Protein Diet
4	Test Substance (1 ng)	14	1.0 µg a.i. IPD072Aa Protein Diet
5	0.5 µg a.i. IPD072Aa Protein Diet	15	1.0 µg a.i. IPD072Aa Protein Diet
6	0.5 µg a.i. IPD072Aa Protein Diet	16	1X LDS/DTT Sample Buffer Blank
7	0.5 µg a.i. IPD072Aa Protein Diet	17	Control Diet Sample
8	1X LDS/DTT Sample Buffer Blank	18	Control Diet Sample
9	1X LDS/DTT Sample Buffer Blank	19	Control Diet Sample
10	Test Substance (10 ng)	20	1X LDS/DTT Sample Buffer Blank

Note: Microgram (µg), active ingredient (a.i.), and lithium dodecyl sulfate with dithiothreitol (LDS/DTT). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Control samples were loaded at the same dilution as the 0.5 µg diet samples. Dose confirmation samples were loaded to a target of 5 ng based on the nominal concentration of IPD072Aa protein in each respective diet.

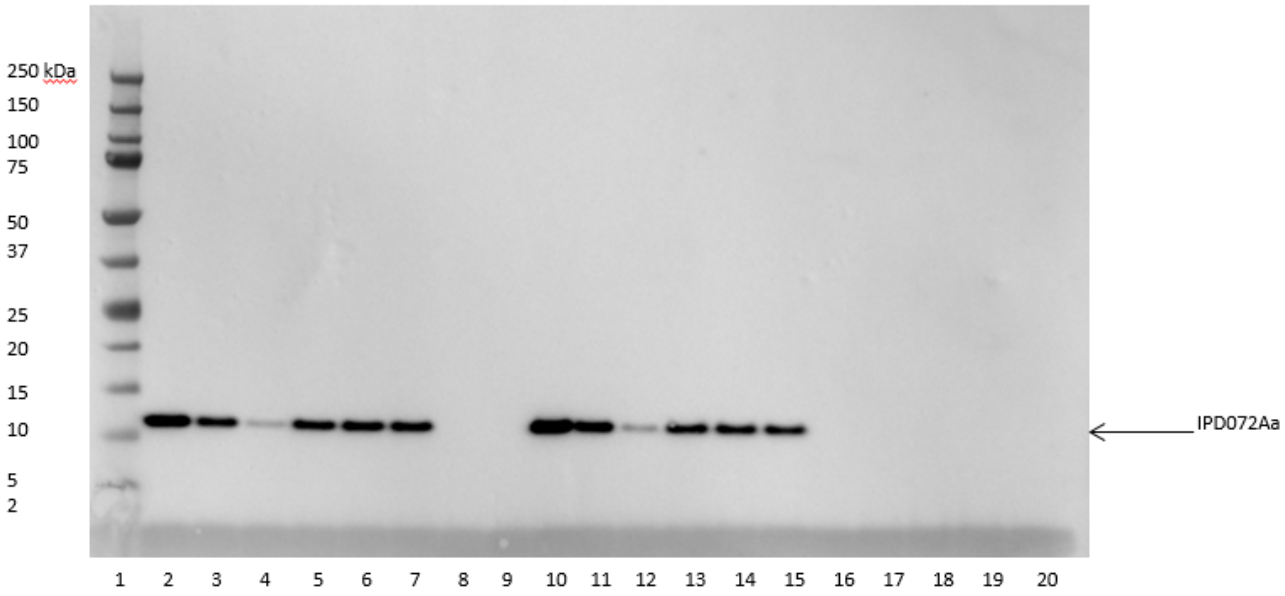
Figure 85. Dose Confirmation of the IPD072Aa Protein in Adult Diets and Assessment of the Absence of IPD072Aa Protein in Control Diet, Day 2



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Test Substance (5 ng)
2	Test Substance (10 ng)	12	Test Substance (1 ng)
3	Test Substance (5 ng)	13	1.0 µg a.i. IPD072Aa Protein Diet
4	Test Substance (1 ng)	14	1.0 µg a.i. IPD072Aa Protein Diet
5	0.5 µg a.i. IPD072Aa Protein Diet	15	1.0 µg a.i. IPD072Aa Protein Diet
6	0.5 µg a.i. IPD072Aa Protein Diet	16	1X LDS/DTT Sample Buffer Blank
7	0.5 µg a.i. IPD072Aa Protein Diet	17	Control Diet Sample
8	1X LDS/DTT Sample Buffer Blank	18	Control Diet Sample
9	1X LDS/DTT Sample Buffer Blank	19	Control Diet Sample
10	Test Substance (10 ng)	20	1X LDS/DTT Sample Buffer Blank

Note: Nanogram (ng), microgram (µg), active ingredient (a.i.), and lithium dodecyl sulfate with dithiothreitol (LDS/DTT). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Control samples were loaded at the same dilution as the 0.5 µg diet samples. Dose confirmation samples were loaded to a target of 5 ng based on the nominal concentration of IPD072Aa protein in each respective diet.

Figure 86. Dose Confirmation of the IPD072Aa Protein in Adult Diets and Assessment of the Absence of IPD072Aa Protein in Control Diet, Day 6



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Test Substance (5 ng)
2	Test Substance (10 ng)	12	Test Substance (1 ng)
3	Test Substance (5 ng)	13	1.0 µg a.i. IPD072Aa Protein Diet
4	Test Substance (1 ng)	14	1.0 µg a.i. IPD072Aa Protein Diet
5	0.5 µg a.i. IPD072Aa Protein Diet	15	1.0 µg a.i. IPD072Aa Protein Diet
6	0.5 µg a.i. IPD072Aa Protein Diet	16	1X LDS/DTT Sample Buffer Blank
7	0.5 µg a.i. IPD072Aa Protein Diet	17	Control Diet Sample
8	1X LDS/DTT Sample Buffer Blank	18	Control Diet Sample
9	1X LDS/DTT Sample Buffer Blank	19	Control Diet Sample
10	Test Substance (10 ng)	20	1X LDS/DTT Sample Buffer Blank

Note: Microgram (µg), active ingredient (a.i.), and lithium dodecyl sulfate with dithiothreitol (LDS/DTT). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Control samples were loaded at the same dilution as the 0.5 µg diet samples. Dose confirmation samples were loaded to a target of 5 ng based on the nominal concentration of IPD072Aa protein in each respective diet.

Figure 87. Dose Confirmation of the IPD072Aa Protein in Adult Diets and Assessment of the Absence of IPD072Aa Protein in Control Diet, Day 9

G2.a. Honey Bee IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of IPD072Aa protein in the stored working stock solution used to create larval and adult honey bee diets was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after return of the stored working stock solution by the CRO following final use in the honey bee bioassays.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (prepared with ultrapure water)

Treatment B: Test Diet (prepared with stored IPD072Aa working stock solution and targeting 393.25 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

On each day of diet preparation, an aliquot of the stored IPD072Aa working stock solution returned from The CRO was removed from frozen storage (-80 °C freezer unit), and thawed on wet ice. Treatments A and B for the WCR bioassay were prepared as follows:

For Treatment A, ultrapure water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment B, stored IPD072Aa protein working stock solution was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed.

The bioassay acceptability criteria indicated a bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented inTable 192.

G3. IPD072Aa Protein: 10-Day Oral Toxicity Test with the Adult Honey Bee (Apis mellifera)

MATERIALS AND METHODS

Protocol

The methods described in this protocol are based on the testing requirements of the OECD 245 Guideline for honey bee (*Apis mellifera L.*), chronic oral toxicity test (10-day feeding), (OECD, 2017), and recent input from the U.S. EPA.

Test Substance

The test substance consisted of IPD072Aa protein. Stability was certified under -80 °C freezer unit storage condition.

The IPD072Aa protein working stock solution was received in solution on 24 May 2018 from Pioneer Hi-Bred International, Inc., Johnston, Iowa.

Upon receipt at the contract research organization (CRO), the IPD072Aa protein Working Stock Solution (No. 9445) was stored in a freezer (-80 °C) in the original container.

Test Organism

The honey bees (*Apis mellifera*) used during this study were ≤2-day old emerged adult bees removed from isolated brood frames containing known aged bees from hives provided by Wood's Beekeeping Supply, Lincoln, Rhode Island. The source hives are identified as Italian hybrids as they contain a mixture of the original subspecies, *A. mellifera ligustica*, and unidentified subspecies, which commonly co-exist in commercial bee hives. The honey bees were from hives that had not previously been exposed to chemical application for at least one month prior to use in testing. Brood frames containing capped brood with no emerged adult bees present were received three days prior to acclimation and age synchronized one day prior to acclimation. Bees that emerged from the brood frames were less than one day old from emergence when manually selected and impartially placed in acclimation vessels. The honey bees were transferred into the test vessels by inserting a bee into the test vessel one at a time until ten bees were added. Vessels containing organisms were then placed into an environmental chamber and acclimated to test conditions until exposure to the dosed diets on the following day. During the 1-day acclimation period, the test organisms were supplied with approximately 2.0 mL of 50% untreated sucrose solution diet, prepared by dissolving an equal

portion of food-grade sucrose in sterile deionized water (w/w). The sucrose solution diet was provided in a syringe inserted through the top of the vessel.

Representative samples of 50% sucrose solution (comprised of water and organic sucrose) were analyzed periodically for the presence of PCBs and selected toxic metals by Eurofins Lancaster Laboratories Environmental, Lancaster, Pennsylvania (US-EPA, 1997). Representative samples of 50% sucrose solution were analyzed for the presence of pesticides by the U.S. Department of Agriculture, Gastonia, North Carolina. Each analyte was considered to be of acceptable quality since no analytes were measured at concentrations considered to be toxic to the test organisms (ASTM, 2007).

Test Vessels

Test vessels were 120-mL glass jars with screw top lids. Ventilation holes and a larger hole for insertion of the feeding syringe were drilled in the lid.

Replication

Three replicate vessels (1, 2, and 3) were established for each treatment level and control group. Each replicate contained 10 honey bees (30 honey bees per treatment level and control). Each replicate vessel was randomly assigned to a treatment or control group using a random number generator prior to the start of the exposure. Each jar was labeled to identify the dose level or control, replicate identification, and study number.

Test Conditions

The 10-day exposure was conducted in an environmental chamber designed to maintain a temperature of 33 ± 2 °C and a relative humidity ranging from 50 to 70%. The environmental chamber maintained the test organisms in near darkness to approximate hive conditions. Honey bees were exposed to laboratory lighting for approximately 30 minutes each day during observations and renewal of sucrose diets.

Test Concentration Selection

Based on consultation with Pioneer, target daily diet concentrations of 20 and 41 mg a.i./kg diet were used to calculate nominal doses of 0.50 and 1.0 µg a.i./bee/day. These nominal doses and a control were chosen for the definitive test.

Preparation of Diets

Test Substance

A 0.55 mg a.i./mL IPD072Aa protein working stock solution was prepared by Pioneer for incorporation into 50% sucrose solution diet. On a daily basis, an aliquot of stock solution was removed from storage and thawed at 4 ± 3 °C in order to dose diets daily for days 0 through 9. A measured amount of stock solution was added to a batch of the appropriate diet.

Diet solutions were mixed with a stir bar and stir plate for at least 15 minutes. All resulting diet solutions were observed each day to be clear and amber in color due to the sucrose in solution with no visible undissolved material.

The control vessels received only fresh, untreated, sucrose solution.

Reference Toxicant

In order to assess the sensitivity of the bees used in testing, a reference test was included in the test design and was conducted concurrently with the definitive exposure using honey bees obtained from the same hives as those used to initiate the definitive test. Dimethoate, a known honey bee toxicant, was used in the reference test.

A 20 mg a.i./mL primary stock solution was prepared by bringing 2.0295 g of dimethoate (2.0254 g as active ingredient) to a volume of 100 mL with acetone (CAS No. 67-64-1). The resulting 20 mg a.i./mL reference toxicant solution was observed to be clear and colorless with no visible undissolved material following initial sonication of 20 seconds and mixing with a stir bar and magnetic stir plate for 10 minutes. A 0.062 mg a.i./mL was prepared by adding 0.31 mL of the 20 mg a.i./mL reference toxicant stock solution to a 100-mL volumetric flask and bringing it to volume with acetone. A single treated diet was prepared using the following preparation scheme:

Primary Stock Concentration (mg a.i./mL)	Volume of Primary Stock Solution Used (mL)	Volume Diluted to with Acetone (mL)	Secondary Stock Concentration (mg a.i./mL)	Volume of Stock Used (mL)	Volume Diluted to with 50% Sucrose Diet (mL)	Final Diet Concentration	
						Nominal Diet Concentration (mg a.i./kg) [a]	Nominal Dose (µg a.i./bee/day) [b]
20	0.31	100	0.062	0.50	25	1.0	0.025

[a] 0.00124 mg a.i.//mL (1.0 mg a.i./kg) based on a 50% Sucrose solution density of 1232.02 mg/mL (USDA, 1981).
[b] Theoretical value based on an estimated consumption rate of 200 µL (246 mg) per 10 bees per day.

NOTE: Results were calculated using the actual unrounded results and not the rounded values presented in this table.

The diet solution was mixed using a stir bar and stir plate for at least 20 minutes and for the diet solution prepared on day 0, sonicated for approximately 15 seconds immediately after dosing and right before feeding. The resulting reference toxicant diet was observed to be clear and amber in color with no visible undissolved material following preparation. The final nominal diet concentration was 1.0 mg a.i./kg of diet solution, equivalent to a dose rate of 0.025 µg a.i./bee/day. This diet solution was prepared on test days 0, 3, and 7 and used for the test days as referenced in the table below:

Day of Secondary Diet Preparation	Days of Use in Testing
0	0 - 2
3	3 - 6
7	7 - 9

Experimental Initiation

The theoretical feeding rate of 200 µL per 10 bees per day was assumed based on past consumption rates and published data (Decourtye *et al.*, 2005). Complete consumption of 200 µL per vessel per day along with actual diet concentrations that closely approximated nominal values would provide the expected nominal dose. Sucrose solution diet was administered to each test vessel using a 3.0-mL plastic syringe with the tip removed, containing approximately 2.0 mL of the appropriate diet. Syringes were filled with the appropriate diet on a daily basis. The actual amount of diet consumed per vessel was verified throughout the test by weighing each syringe before and after each daily feeding to the nearest 0.0001 g using a Sartorius (Secura 225D-1S) analytical balance. The following table details how diet concentrations were used to derive each dose rate:

Final Diet Concentration (mg a.i./kg)	Theoretical Amount Consumed per Vessel (µL/day)[a]	Number of Bees per Vessel	Nominal Dose Rate per Bee (µg a.i./bee/day)
20	200	10	0.50
41	200	10	1.0

[a] 50% Sucrose solution density = 1232.02 mg/mL (USDA, 1981).

NOTE: Results were calculated using the actual unrounded results and not the rounded values presented in this table.

Each feeding syringe and vessel was labeled to identify the concentration, replicate, and study number. Bees were allowed to feed on the diets *ad libitum* each day during the 10-day exposure.

Test Monitoring

Observations of the honey bees exposed to IPD072Aa Protein and the controls were made daily. Mortality and any unusual behavior exhibited by the bees (e.g., apathy) were also recorded. Test organisms were considered dead if observed to be immobile on the bottom of the vessel, exhibiting no response to gentle prodding. At termination (day 10), all surviving bees were frozen and weighed on a Sartorius (Secura 225D-1S) analytical balance to the nearest 0.0001 g.

Feeding syringes containing diets were weighed daily before and after filling with freshly prepared diet solution in a similar manner as described below. Additionally, three vessels were established, each with one syringe filled with untreated sucrose solution and without honey bees, and the syringe weight monitored daily for evaporative loss. Daily honey bee dose was then calculated from daily diet consumption, daily evaporative loss, and daily mortality to more accurately reflect the daily dose. Daily dose was calculated using the following formula:

$$Daily\ dose\ (\frac{\mu g}{bee}) = \left(\frac{mg\ food\ consumed}{bee} \times \frac{1\ mL}{1232.02\ mg\ *}\right) \times nominal\ diet\ concentration\ (\frac{\mu g}{mL})$$

* 50% Sucrose solution density = 1232.02 mg/mL (USDA, 1981).

NOTE: The amount of diet weight loss caused by evaporation is subtracted from the syringe weight difference over the feeding period before reported as mg food consumed.

Relative humidity within the environmental chamber and continuous temperature measurements were monitored continuously using a HOBO data logger (Onset Computer Corporation, Model ZW-007).

Analytical Measurements

Analytical measurements were conducted by Pioneer. Five samples of test and control diet were collected on days 2, 6, and 9 of the exposure. In addition, all remaining aliquots of the stock solution were returned to Pioneer. Results of these analyses were used to confirm that the stock solutions and treated diets were correctly prepared and to quantify actual exposure concentrations.

All samples were stored at -80 ± 10°C prior to shipping. Samples were shipped on dry ice to Pioneer and were held frozen until analysis.

Determination of the NOEC/NOEDD and LOEC/LOEDD Values

The endpoints for this study are expressed as nominal diet concentration (mg a.i./kg) and calculated mean daily dose (µg a.i./bee/day). The highest test concentration in diet and dietary dose that elicited no statistically significant reduction between the exposed organisms and the appropriate control (No-Observed-Effect Concentration, NOEC, and No-Observed-Effect Dietary Dose, NOEDD) were estimated. The lowest test concentration in diet and dietary dose that elicited a statistically significant reduction on organism performance (Lowest-Observed-Effect Concentration, LOEC, and Lowest-Observed-Effect Dietary Dose, LOEDD) were also estimated. The treatment data were tested for normality and homogeneity of variance using the appropriate qualifying test. Basic guidance for this determination is as follows, but the actual analysis used is dependent on the data set:

Endpoint	Normal Distribution[a]	Equal Variance[b]	Statistical Test
Percent Survival	Not Applicable	Not Applicable	**Monotonic Trend:** Cochran-Armitage's Step-Down Test **Non-Monotonic Trend:** Fisher's Exact Test with Bonferroni-Holm's Adjustment
Weight	Yes	Yes	**Monotonic Trend:** Williams' Multiple Comparison Test or Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunnett's Multiple Comparison Test
	No	Yes or No	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Dunn's Test or Wilcoxon's Test with Bonferroni-Holm's Adjustment
	Yes	No	**Monotonic Trend:** Jonckheere-Terpstra's Step-Down Test **Non-Monotonic Trend:** Tamhane-Dunnett's Test or Dunnett's T3 Test

[a] Shapiro-Wilks' Test was used to assess normality of variance.
[b] Bartlett's Test was used to assess homoscedasticity.

CETIS Version 1.8 was used to determine the NOEC/NOEDD and LOEC/LOEDD values.

RESULTS

Environmental Conditions

The temperature monitored within the environmental chamber ranged from 33 to 34°C and the relative humidity ranged from 59 to 68% during the study. Based on historical data, these conditions were within an acceptable range for honey bee survival.

Analytical Results

The diet characterization for each treatment was performed by Pioneer. As the analysis confirmed nominal concentrations, results are reported as nominal values.

Biological Results

IPD072Aa Protein Exposure

Based on feeding syringe weights recorded at the beginning and end of each exposure interval, the following mean amount of diet consumed was 30, 32, and 31 mg/bee/day for the control and 0.50 and 1.0 µg a.i./bee/day calculated mean daily dose rates, respectively. Food consumption by treatment is presented in Table 193. These data indicate that the diet consumption in the dose rates were very similar to the control consumption rates.

Table 194 presents the calculated mean daily dose rates tested and corresponding daily percent survival and day 10 percent mortality observed during the 10-day oral exposure.

Following 10 days of exposure, a percent mortality of 10% was observed among honey bees exposed to both the 0.50 and 1.0 µg a.i./bee/day treatment. A percent mortality of 3% was observed among the control honey bees. Fisher's Exact Test with Bonferroni Holm's Adjustment indicated no significant mortality in any of the dose rates relative to the control. The 10-day NOEC and NOEDD value for mortality were determined to be 41 mg a.i./kg and 1.3 µg a.i./bee/day, respectively (Table 197). The 10-day LOEC and LOEDD value for mortality was determined to be >41 mg a.i./kg and >1.3 µg a.i./bee/day, respectively (Table 197).

Table 196 presents the calculated mean daily dose rates tested and corresponding mean body weights measured following the 10-day oral exposure. Mean body weight of 0.0993 and 0.1099 g was observed among honey bees exposed to the test treatments (0.50 and 1.0 µg a.i./bee/day dose rates, respectively). Mean weight of 0.1042 g was observed among the control honey bees. The 10-day NOEC and NOEDD values for weight were determined to be 41 mg a.i./kg diet and 1.3 µg a.i./bee/day, respectively (Table 197). The 10-day LOEC and LOEDD values for weight were determined to be >41 mg a.i./kg diet and >1.3 µg a.i./bee/day, respectively (Table 197).

Dimethoate Exposure (Reference Test)

Table 195 presents the results of the biological exposure. The nominal dose of dimethoate maintained during the reference test was 0.025 µg a.i./bee/day. Procedures used during the reference test were consistent with the procedures used in the definitive test. The percent mortality on day 10 was 100% in the 0.025 µg a.i./bee/day nominal dose. The results demonstrate that the honey bees were sensitive to dimethoate in this exposure system and the mortality was >50% at this dose as expected in the draft guideline.

ACCEPTABILITY CRITERIA

The following acceptance criteria were required:

Acceptability Criteria	Study Results	Criterion Met
Mean mortality in the control must not equal or exceed 15% at termination.	Cumulative percent mortality in the control was 3% at test termination.	Yes
Mean mortality in the reference test must be ≥50% after 10 days of exposure.	Percent mortality of 100% was observed during the dimethoate reference test.	Yes

CONCLUSIONS

The 10-day percent survival NOEDD and LOEDD values for IPD072Aa Protein to honey bees were determined to be 1.3 and >1.3 µg a.i./bee/day, respectively. The 10-day honey bee weight NOEDD and LOEDD values for IPD072Aa Protein to honey bees were determined to be 1.3 and >1.3 µg a.i./bee/day, respectively.

Table 197 summarizes the established endpoints for this study (NOEC/NOEDD and LOEC/LOEDD values) based on nominal diet concentrations and calculated mean daily dose. Results of the toxic reference standard test (dimethoate) indicated that the test organisms were responsive to a toxicant in this study design.

Table 193. 10-Day Oral Exposure of Honey Bees (*Apis mellifera*) to IPD072Aa Protein - Food Consumption, Calculated Mean Daily Dose, and Accumulated Dose

Nominal Dose (µg a.i./bee/day)	Nominal Diet Concentration (mg a.i./kg)	Overall Mean Daily Consumption of Food Solution (mg/bee/day)	Calculated Mean Daily Dose (µg a.i./bee/day)[a b]	Mean Accumulated Dose (µg a.i./bee)
Control	NA [c]	30	NA	NA
0.50	20	32	0.64	6.4
1.0	41	31	1.3	13

[a] Daily honey bee diet consumption was corrected for average, daily evaporative loss (overall mean daily evaporation was 58 mg).
[b] Calculated mean daily dose (µg a.i./bee/day) = mean diet consumed (mg/bee) × nominal concentration (mg a.i./kg).
[c] NA = Not Applicable

NOTE: Values were calculated from the raw data and not the rounded values presented in this table.

Table 194. 10-Day Oral Exposure of Honey Bees (*Apis mellifera*) to IPD072Aa Protein - Survival and Mortality

Calculated Mean Daily Dose (µg a.i./bee/day)	Replicate	Percent Survival										Day 10	
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Cumulative Percent Mortality Day 10	Abbott's Corrected Percent Mortality[a]
Control	1	100	100	100	100	100	100	100	100	100	100	0	
	2	100	100	100	100	100	90	90	90	90	90	10	
	3	100	100	100	100	100	100	100	100	100	100	0	
	Mean (SD[b])	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**97 (6)**	**97 (6)**	**97 (6)**	**97 (6)**	**97 (6)**	**3 (6)**	**NA[c]**
0.64	1	100	100	100	100	100	100	100	100	100	100	0	
	2	100	100	100	100	100	100	100	100	90	90	10	
	3	90	90	90	90	90	90	90	90	90	80	20	
	Mean (SD)	**97 (6)**	**97 (6)**	**97 (6)**	**97 (6)**	**97 (6)**	**97 (6)**	**97 (6)**	**97 (6)**	**93 (6)**	**90 (10)**	**10 (10)**	**7**
1.3	1	100	100	100	100	100	100	100	100	100	80	20	
	2	100	100	100	100	100	100	100	90	90	90	10	
	3	100	100	100	100	100	100	100	100	100	100	0	
	Mean (SD)	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**100 (0)**	**97 (6)**	**97 (6)**	**90 (10)**	**10 (10)**	**7**

[a] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925). Statistical analysis was conducted using uncorrected values.

[b] SD = Standard Deviation

[c] NA = Not Applicable

NOTE: A total of 30 organisms per dose or control were exposed at test initiation.

Table 195. 10-Day Oral Exposure of Honey Bees (*Apis mellifera*) to the Reference Toxicant, Dimethoate - Mortality
Dimethoate Reference Test

Nominal Daily Dose (µg a.i./bee/day)[a]	Cumulative Percent Survival (Number of Dead Larvae)									Day 10	
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Percent Mortality (Number of Dead Larvae)	Abbott's Corrected Percent Mortality[b]
0.025	97 (1)	97 (1)	97 (1)	90 (3)	57 (13)	23 (23)	7 (28)	3 (29)	0 (30)	100 (30)	100

[a] Equivalent to 1200 µg a.i./L of diet, or 1.0 mg a.i./kg of diet.
[b] Per the study guideline, mortality values were corrected using Abbott's formula (Abbott, 1925).

NOTE: A total of 30 organisms per dose were exposed at test initiation.

Table 196. 10-Day Oral Exposure of Honey Bees (Apis mellifera) to IPD072Aa Protein - Weight

Calculated Mean Daily Dose (µg a.i./bee/day)	N	Mean Weight, g (SD[a])
Control	29	0.1042 (0.0013)
0.64	27	0.0993 (0.0060)
1.3	27	0.1099 (0.0076)

[a] SD = Standard Deviation

Table 197. 10-Day Oral Exposure of Honey Bees (*Apis mellifera*) to IPD072Aa Protein - Endpoint Summary

Endpoint	Nominal Diet (mg a.i./kg)	Calculated Mean Daily Dose (µg a.i./bee/day)
	Percent Survival	
NOEC/NOEDD	41	1.3
LOEC/LOEDD	>41	>1.3
	Live Adult Weight	
NOEC/NOEDD	41	1.3
LOEC/LOEDD	>41	>1.3

G4. Evaluation of the Survival of Parasitic Hymenoptera Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unitstorage condition.

Carrier

The carrier consisted of 30% sucrose prepared weight by volume (w/v) with ultrapure water.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the targeted concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section G4.a. Parasitic Hymenoptera IPD072Aa Protein Bioassay Analytical Phase

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was Pediobius foveolatus (Hymenoptera: Eulophidae). Parasitic Hymenoptera was selected as a representative non-target organism to characterize IPD072Aa protein. Parasitic Hymenoptera pupae were obtained from State of New Jersey Department of Agriculture (Trenton, NJ, USA) and identity was recorded by study personnel.

Experimental Design

Parasitic Hymenoptera adults were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of sucrose in ultrapure water)
- Treatment 2: Test Diet (targeting 100 µg IPD072Aa protein per ml sucrose diet)
- Treatment 3: Test Diet (targeting 500 µg IPD072Aa protein per ml sucrose diet)
- Treatment 4: Test Diet (targeting 1000 µg IPD072Aa protein per ml sucrose diet)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 µg heat-treated IPD072Aa protein per ml sucrose diet)
- Treatment 6: Positive Control Diet (targeting 20,000 µg boric acid per ml sucrose diet)

Treatments were arranged in a generalized randomized block design with a total of 6 blocks. Each block consisted of 1-oz plastic cups in a 30-well tray and contained 5 replicates from each treatment. Each treatment was provided to a target of 30 Parasitic Hymenoptera individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Adults were refed every other day. After 7 days, the bioassay was complete and mortality was assessed. Mortality was statistically compared between Parasitic Hymenoptera provided Treatment 1 and those provided Treatment 2, 3, or 4.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%.

An enzyme-linked immunosorbent assay (ELISA) was used to assess the dose of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay and frozen storage conditions of the IPD072Aa protein in Treatment 2. The presence or absence of IPD072Aa protein in Treatments 1 and 5 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the Parasitic Hymenoptera bioassay.

Bias in the Parasitic Hymenoptera bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing and appropriate assay controls.

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-5 were prepared as described in the Analytical Phase section below. Each dosing solution was mixed with 60% sucrose (w/v) in a 1:1 ratio (*i.e.*, 1 ml of dosing solution to 1 ml of sucrose solution), generating Treatments 1-5. Treatment 6 was prepared by diluting boric acid stock solution in ultrapure water and mixing with 60% sucrose in a 1:1 ratio. All diets were prepared to a final concentration of 30% sucrose. After mixing, diets were aliquoted into individual microcentrifuge tubes (200 µl per tube) for the duration of the bioassay and a square of autoclaved tulle was added to each tube. Tubes were closed and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1-5 were collected for characterization as described in the Analytical Phase section below. In addition, a portion each of Treatment 1 and Treatment 4 was collected for use in diets for the sensitive insect bioassay as described in section G4.b. Parasitic Hymenoptera IPD072Aa Protein Sensitive Insect Bioassay.

The positive control diet (Treatment 6) was not characterized.

Parasitic Hymenoptera Bioassay

Parasitic Hymenoptera pupae were incubated until adult emergence. Adults were used in the bioassay within 48 hours of emergence.

On Day 0, diet aliquots were removed from frozen storage, uncapped, and distributed into individual plastic cups. One Parasitic Hymenoptera adult was placed in each cup and the cup was sealed with a lid. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for 7 days. Every other day, trays were removed from the environmental chamber and missing or dead organisms were recorded. Tubes containing diet were replaced with new aliquots as described for Day 0 and the trays were returned to the environmental chamber. After 7 days, the bioassay was complete and mortality was assessed. Only cups that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a cup were excluded from statistical analysis.

Statistical Analysis

Statistical analysis of data was conducted using SAS software, Version 9.4. Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of Parasitic Hymenoptera fed the artificial insect diet containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Demonstration of IPD072Aa Protein Activity in a *Parasitic Hymenoptera* Test Diet

A portion of Treatments 1 and 4 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay using WCR larvae to demonstrate the biological activity of the IPD072Aa protein in Treatment 4 of the Parasitic Hymenoptera bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section.

Results and Discussion

The Parasitic Hymenoptera bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 198. The mortality of Parasitic Hymenoptera fed the test diets containing 100 µg/ml, 500 µg/ml, and 1000 µg/ml IPD072Aa protein (Treatments 2, 3, and 4, respectively) was not significantly greater than those fed the bioassay control diet (Treatment 1), as shown in Table 198. Mortality was 17.2% in Treatment 2 (P-value = 0.0896), 16.7% in Treatment 3 (P-value = 0.0973), and 20.0% in Treatment 4 (P-value = 0.0514). Mortality was 3.33% in Treatment 1.

ELISA analysis assessed the dose of IPD072Aa protein in Treatments 2, 3, and 4 (Table 200; 100%, 132%, and 136% of target, respectively). The doses in Treatments 3 and 4 were above the acceptable range of 70-130% of target (Table 200); however, this was determined not to impact the study as there was no statistically significant difference observed in any of the test treatments. In addition, the bioassay and freezer stability of the IPD072Aa protein in Treatment 2 (Table 200; Table 201) and the presence or absence of IPD072Aa protein in Treatments 1 and 5 were also assessed (Table 200). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5) but not in the bioassay control diet (Treatment 1). Homogeneity was not analyzed because the diets were in solution.

Observed larval mortality for the WCR sensitive insect bioassay is summarized in Table 2. The WCR bioassay met the acceptability criterion (Sensitive Insect Bioassay section). The biological activity of the IPD072Aa protein in Treatment 4 of the Parasitic Hymenoptera bioassay was demonstrated by 100% mortality of WCR fed the test diet.

Conclusion

The results demonstrated that exposure to a concentration of 100, 500, or 1000 µg IPD072Aa protein per ml (Treatments 2, 3, and 4, respectively) had no adverse effect on survival of Parasitic Hymenoptera.

Table 198. Summary Analysis of Parasitic Hymenoptera IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (µg IPD072Aa/ml)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	30	1	3.33	--
2	Test Diet	100	29[a]	5	17.2	0.0896
3	Test Diet	500	30	5	16.7	0.0973
4	Test Diet	1000	30	6	20.0	0.0514
5	Heat-treated Control Diet	1000	30	5	16.7	--
6	Positive Control Diet	0[b]	30	29	96.7	--

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.
[b] Treatment 6 contained a targeted concentration of 20,000 µg boric acid per ml.

Table 199. Summary of Parasitic Hymenoptera IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0	30	5	16.7
B	Test Diet	357.5	29[a]	29	100

Note: Treatments A and B used in the sensitive insect WCR bioassay were prepared from the same stored diet preparations used in Treatments 1 and 4, respectively, of the Parasitic Hymenoptera bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

[a] Wells that contained more than one organism were not included in the total number of observations for a given treatment.

Table 200. Assessment of IPD072Aa Protein Concentration in Test Diets and Absence in Control Diets

Analysis	Diet	Treatment	Result
Concentration Verification	Test Diet	2	100% of Target[a]
		3	132% of Target[a]
		4	136% of Target[a]
Presence/Absence in Control Diet	Bioassay Control	1	Absence confirmed[b]
	Heat-Treated Control	5	Presence confirmed[b]

[a] Value is the average of three samples. Concentrations within 70-130% of the expected value were considered verified.
[b] Absence of IPD072Aa protein was verified by results less than the lowest standard curve concentration minus 10%.

Table 201. Verification of IPD072Aa Protein Stability in Test Diet (Treatment 2)

Stability under Bioassay Conditions		Frozen Storage Stability	
Bioassay Stability Day	R%D of Day of Prep Mean[a]	Storage Timepoint	R%D of Day of Prep Mean[a]
Diet Preparation Day	NA	Diet Preparation Day	NA
Day 1	9	~1 Week	4
		~2 Weeks	10
		~3 Weeks	11

Note: Frozen storage stability samples were stored in a -80 °C freezer unit. Not applicable (NA).
[a] Value is the average of three samples. Relative stability was calculated by the relative percent difference (R%D) of the stored concentration compared to the concentration measured on the day of preparation.

G4.a. Parasitic Hymenoptera IPD072Aa Protein Bioassay Analytical Phase

The following dosing solutions were prepared for the Parasitic Hymenoptera bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

An enzyme-linked immunosorbent assay (ELISA) was used to assess the dose of the IPD072Aa protein in Treatments 2, 3, and 4 (100 µg, 500 µg, and 1000 µg IPD072Aa protein per ml diet, respectively) and the stability under bioassay conditions and frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively) were also assessed.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation for the *Parasitic Hymenoptera* bioassay and maintained on wet ice until use. To generate the test dosing solutions for Treatments 2, 3, and 4, the test substance was removed from frozen storage, thawed under chilled conditions and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (200 µg/ml, 1000 µg/ml, and 2000 µg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water.

Characterization of Diets

Sample Collection

During the process of diet preparation for the Parasitic Hymenoptera bioassay, diet samples were collected into tubes as shown in Table 202.

Table 202 Parasitic Hymenoptera IPD072Aa Protein Bioassay Diet Samples

IPD072Aa Protein Analysis	Treatment	Number of Samples Collected	Number of Samples Analyzed
Dose confirmation	2	3	3
	3	3	3
	4	3	3
Bioassay conditions stability	2	5	3
Frozen storage stability	2	9	9
Presence/absence	1	1	1
	5	1	1

Note: Treatment 2 dose confirmation samples were used for initial (day of diet preparation) samples for stability under bioassay and frozen storage conditions.

Stability under bioassay conditions samples were distributed as described in Section B.2. below. Dose confirmation and presence/absence samples were immediately prepared for ELISA analysis, as described below. Frozen storage stability samples were stored frozen (-80 °C freezer unit) and at each time point (approximately Weeks 1, 2, and 3), three samples were removed from the freezer and prepared for ELISA analysis.

Stability under Bioassay Conditions

Samples collected during diet preparation were randomly distributed to cups and placed under bioassay conditions using the same methods and conditions used in the *Parasitic Hymenoptera*

bioassay, except they were not infested with larvae. On Day 1, the cups were removed from bioassay conditions and the samples were stored frozen (-80 °C freezer unit) until analysis.

IPD072Aa Protein ELISA Method

The concentration of IPD072Aa protein in each test diet (Treatments 2, 3, and 4), the stability of IPD072Aa protein in Treatment 2 and the absence of IPD072Aa protein in the control diets (Treatments 1 and 5) was assessed using a quantitative ELISA method that had been internally validated to demonstrate method suitability. Samples were interpolated using a standard curve with the IPD072Aa test substance as the analytical standard. Prior to analysis, samples were diluted as applicable in chilled 25% StabilZyme Select in phosphate-buffered saline containing polysorbate 20 (PBST). Standards and diluted samples (analyzed in triplicate wells) were incubated in a plate pre-coated with an IPD072Aa-specific antibody. Following incubation, unbound substances were washed from the plate. A different IPD072Aa-specific antibody, conjugated to the enzyme horseradish peroxidase (HRP), was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound IPD072Aa-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader.

Determination of Protein Concentration

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the OD values obtained for each set of sample wells to a protein concentration value.

A standard curve was included on each ELISA plate. The equation for the standard curve was derived by the software, which used a quadratic fit to relate the OD values obtained for each set of standard wells to the respective standard concentration (ng/ml).

The quadratic regression equation was applied as follows: $y = Cx^2 + Bx + A$

where x = known standard concentration and y = respective absorbance value (OD)

Interpolation of the sample concentration (ng/ml) was performed by solving for x in the above equation using the values for A, B, and C that were determined for the standard curve.

$$\text{Sample Concentration (ng/ml)} = \frac{-B + \sqrt{B^2 - 4C(A - \text{sample OD})}}{2C}$$

For example, given curve parameters of A = 0.0476, B = 0.4556, C= -0.01910, and a sample OD = 1.438

Sample Concentration = $\frac{-0.4556+\sqrt{0.4556^2-4(-0.01910)(0.0476-1.438)}}{2(-0.01910)}$ = 3.6 ng/ml

The sample concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the interpolated concentration by N.

Adjusted Concentration = Interpolated Sample Concentration x Dilution Factor

For example, given an interpolated concentration of 3.6 ng/ml and a dilution factor of 1:20

Adjusted Concentration = 3.6 ng/ml x 20 = 72 ng/ml

Verification of Absence of IPD072Aa Protein in Control Diets

The absence of IPD072Aa protein in control diet samples was verified by concentration results less than the lower limit of quantitation (LLOQ) of each assay plate:

LLOQ = (Mean concentration of lowest Standard Curve Point – 10%)

Verification of IPD072Aa Protein Stability in Treatment 2

Stability was considered verified if the average percentage for a sampling time point was within 70-130% of the mean concentration on the day of diet preparation. Stability was calculated for each stability condition and time point as follows:

$$\text{Relative \% Difference} = \left| \frac{\text{(Day of Preparation Mean Result - Stored Sample Mean Result)}}{\text{Day of Preparation Mean Result}} \right| \times 100$$

G4.b. Parasitic Hymenoptera IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the Pediobius foveolatus (Parasitic Hymenoptera) bioassay was evaluated by conducting a 7-day bioassay using Diabrotica virgifera virgifera (WCR), a species sensitive to IPD072Aa protein. The WCR bioassay was initiated following the final refeed for the Parasitic Hymenoptera bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing a diluted portion of Treatment 1)

Treatment B: Test Diet (containing a diluted portion of Treatment 4 and targeting 357.5 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was provided to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 4 during diet preparation for the Parasitic Hymenoptera bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

Treatments 1 and 4 were diluted with ultrapure (American Society for Testing and Materials ASTM Type 1) water to result in dosing solutions containing 15% sucrose.

Diluted Treatment 1 dosing solution was mixed with carrier in a 2.51:1 ratio (2.51 ml liquid:1 gram of dry diet) to prepare Treatment A.

Diluted Treatment 4 dosing solution was mixed with carrier in a 2.51:1 ratio to prepare Treatment B.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were

dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; wells containing more than one organism were excluded from statistical analysis.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented inTable 199.

G5. Evaluation of the Survival and Development of *Green Lacewing* Fed Artificial Diet Containing IPD072Aa Protein

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier consisted of a meat-based diet prepared by Pioneer Hi-Bred International, Inc.

Positive Control

The positive control consisted of cryolite (AlF_6Na_3).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solution used to prepare Treatment 2 consisted of the test substance diluted in ultrapure water to achieve the concentration in the test diet.

The heat-treated control dosing solution used to prepare Treatment 3 consisted of a portion of the test dosing solution used to prepare Treatment 2 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section G5.a. Green Lacewing IPD072Aa Protein Bioassay Analytical Phase.

Test System

The test system was Green Lacewing (green lacewing; Neuroptera: Chrysopidae). Green Lacewing was selected as a representative non-target organism to characterize IPD072Aa protein. Green Lacewing eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

Green Lacewing larvae were exposed via oral ingestion to one of the following four treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 500 ng IPD072Aa protein per mg diet wet weight)
- Treatment 3: Heat-Treated Control Diet (targeting 500 ng heat-treated IPD072Aa protein per mg diet wet weight)
- Treatment 4: Positive Control Diet (targeting 25,000 ng cryolite per mg diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of 8 blocks. Each block consisted of a tray containing 1-oz plastic cups and contained five replicates from each treatment. Each treatment was provided to a target of 40 Green Lacewing individuals. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed daily and assessed for pupation. On Day 18, all organisms had either pupated or died and the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group.
- The mortality of the positive control diet (Treatment 4) group does not exceed 80%.

Western blot analysis was used to visually confirm the dose, homogeneity, and frozen storage stability of IPD072Aa protein in Treatment 2. The presence or absence of IPD072Aa protein in Treatments 1 and 3 were also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 2 of the Green Lacewing bioassay.

Bias in the Green Lacewing bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-3 were prepared and maintained as described in the Analytical Phase section below. Each dosing solution was mixed with meat-based carrier in a 9:1 ratio (i.e., 9 g carrier to 1 ml dosing solution) to generate Treatments 1-3. Treatment 4 was prepared by mixing cryolite

with carrier and then combining with ultrapure water in a 9:1 ratio to a nominal concentration of 25,000 ng cryolite per mg diet wet weight. After mixing, individual diet packets were prepared for each treatment using an encapsulation device and stored frozen (-20 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1-3 were collected for characterization as described in the Analytical Phase section below. In addition, a portion each of Treatment 1 and Treatment 2 was collected for use in diets for the sensitive insect bioassay and stored frozen (-20 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in section G5.b. Green Lacewing IPD072Aa Protein Sensitive Insect Bioassay.

The positive control diet (Treatment 4) was not characterized.

Green Lacewing Bioassay

Green Lacewing eggs were incubated in an environmental chamber until the eggs hatched. Green Lacewing neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet for each treatment was removed from frozen storage. A moisture source (a tube filled with 0.5% agar) was added to each plastic cup utilized in the bioassay. A small hole was poked into each encapsulated diet packet and one thawed diet packet was placed in each plastic cup. A target of one Green Lacewing neonate was placed in each cup containing diet, and then the cup was secured with a lid. Due to low hatching, only 35 neonates were available in Treatment 4. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for 18 days. Trays were removed from the environmental chamber and pupated, missing, or dead organisms were recorded daily. Diet packets were replaced with new aliquots as described for Day 0 and the trays were returned to the environmental chamber. After 18 days, the bioassay was complete and final mortality was assessed. Only cups that contained one organism were included in the total number of observed individuals; organisms recorded as lost in transfer were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of

Green Lacewing fed the artificial insect diet containing IPD072Aa protein (m_T) (Treatment 2) was greater than the mortality rate of those fed the bioassay control diet (m_C) (Treatment 1). The corresponding hypothesis test was

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Rate of Pupation

Rate of pupation was calculated as percentage of pupated insects out of survived insects. Statistical comparison was not conducted due to 100% pupation in both Treatment 1 and Treatment 2.

Demonstration of IPD072Aa Protein Activity in the Green Lacewing Test Diet

A portion of Treatments 1 and 2 was used to prepare Treatments A and B, respectively, in the sensitive insect bioassay using WCR larvae to demonstrate the biological activity of the IPD072Aa protein in Treatment 2 in the Green Lacewing bioassay. Details regarding the sensitive insect bioassay are provided in the Sensitive Insect Bioassay section below.

Results and Discussion

The Green Lacewing bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 4) group exceeded 80%, as shown in Table 203.

The mortality of Green Lacewing fed the test diet containing 500 ng IPD072Aa protein per mg diet (Treatment 2; 10.3% mortality; P-value = 0.7465) was not significantly greater than those fed the bioassay control diet (Treatment 1; 12.5% mortality), as shown in Table 203. Observed pupation was 100% in both treatments (Table 204).

Western blot analysis visually confirmed the dose, homogeneity, and frozen storage stability over four weeks of the IPD072Aa protein in Treatment 2 (Figure 88; Figure 89). The presence or absence of IPD072Aa protein in Treatments 1 and 3 were also assessed (Figure 90). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 3, Figure 90); however, the band was less intense than that of the test substance. No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 90).

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 3. The WCR bioassay met the acceptability criterion (the Sensitive Insect Bioassay section below). The biological activity of the IPD072Aa protein in Treatment 2 of the Green Lacewing bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in the Sensitive Insect Bioassay section below) when compared to those fed the bioassay control diet (Treatment A).

Conclusion

The results demonstrated exposure to a concentration of 500 ng IPD072Aa protein per mg diet had no adverse effect on mortality or pupation of Green Lacewing.

Table 203. Summary Analysis of Green Lacewing IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Exact Test P-Value
1	Bioassay Control Diet	0	40	5	12.5	--
2	Test Diet	500	39[a]	4	10.3	0.7465
3	Heat-treated Control Diet	500	40	2	5.00	--
4	Positive Control Diet	0[b]	35[c]	35	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet wet weight.
[a] Organisms lost in transfer were not included in the total number of observations for a given treatment.
[b] Treatment 4 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight.
[c] Treatment 4 was infested with 35 organisms due to lower than expected insect hatching.

Table 204. Summary of Green Lacewing IPD072Aa Protein Bioassay Pupation Results

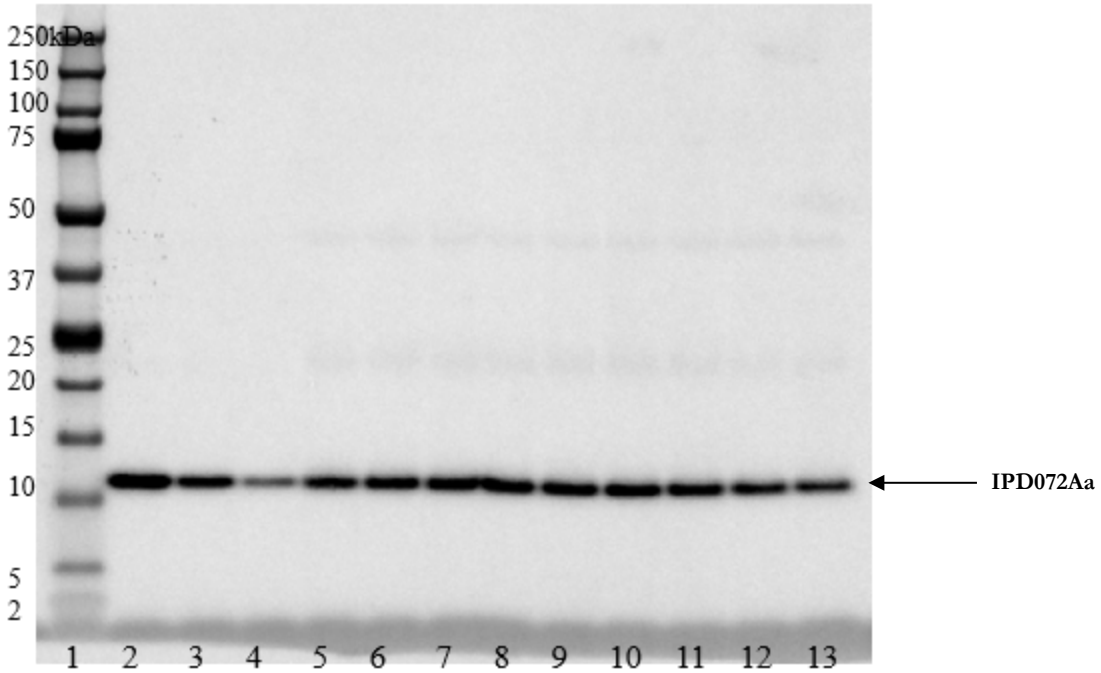
Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Alive Larvae	Number of Pupated Larvae	Pupation (%)
1	Bioassay Control Diet	0	35	35	100
2	Test Diet	500	35	35	100
3	Heat-treated Control Diet	500	38	38	100
4	Positive Control Diet	0[a]	0	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2 and 3 were based on diet wet weight.
[a] Treatment 4 contained a targeted concentration of 25,000 ng cryolite per mg diet wet weight.

Table 205. Summary of Green Lacewing IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	30	10.0	27	0.433 ± 0.121	0.1 - 0.7
B	Test Diet	100	30	70.0	9	0.0889 ± 0.0601	0.0 - 0.2

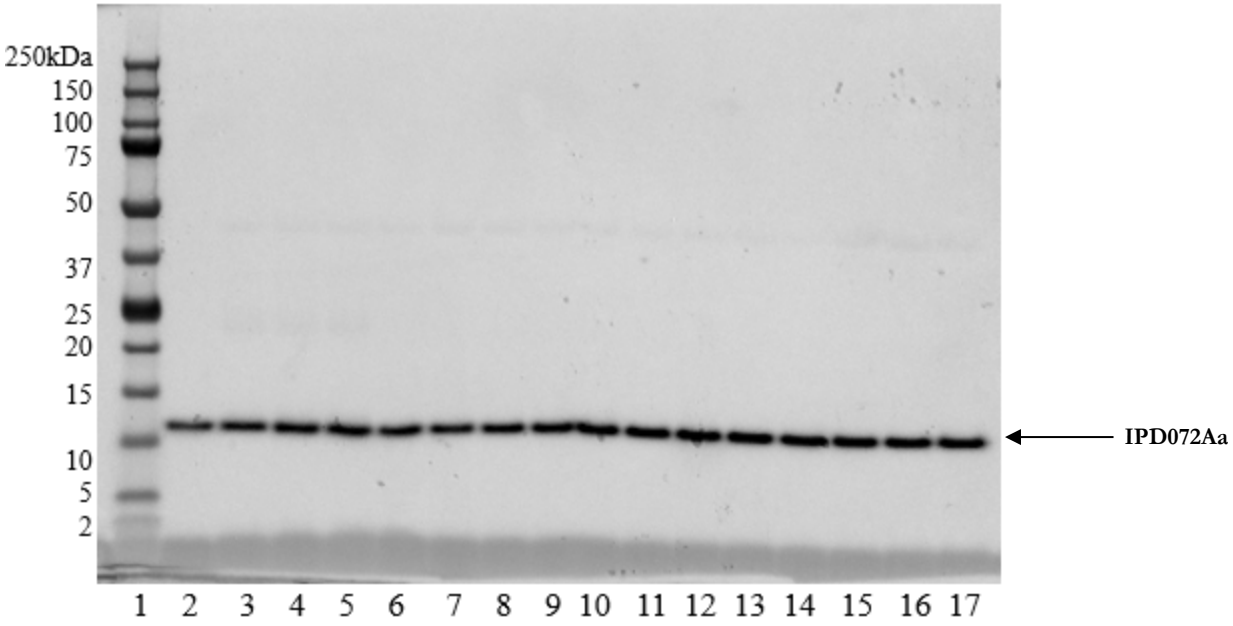
Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 2, respectively, of the *Green Lacewing* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	IPD072Aa Protein Test Diet (Treatment 2; Dose Confirmation, Homogeneity Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Dose Confirmation, Homogeneity Beginning)
7	IPD072Aa Protein Test Diet (Treatment 2; Homogeneity Beginning)
8	IPD072Aa Protein Test Diet (Treatment 2; Homogeneity Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; Homogeneity Middle)
10	IPD072Aa Protein Test Diet (Treatment 2; Homogeneity Middle)
11	IPD072Aa Protein Test Diet (Treatment 2; Homogeneity End)
12	IPD072Aa Protein Test Diet (Treatment 2; Homogeneity End)
13	IPD072Aa Protein Test Diet (Treatment 2; Homogeneity End)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Diet samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

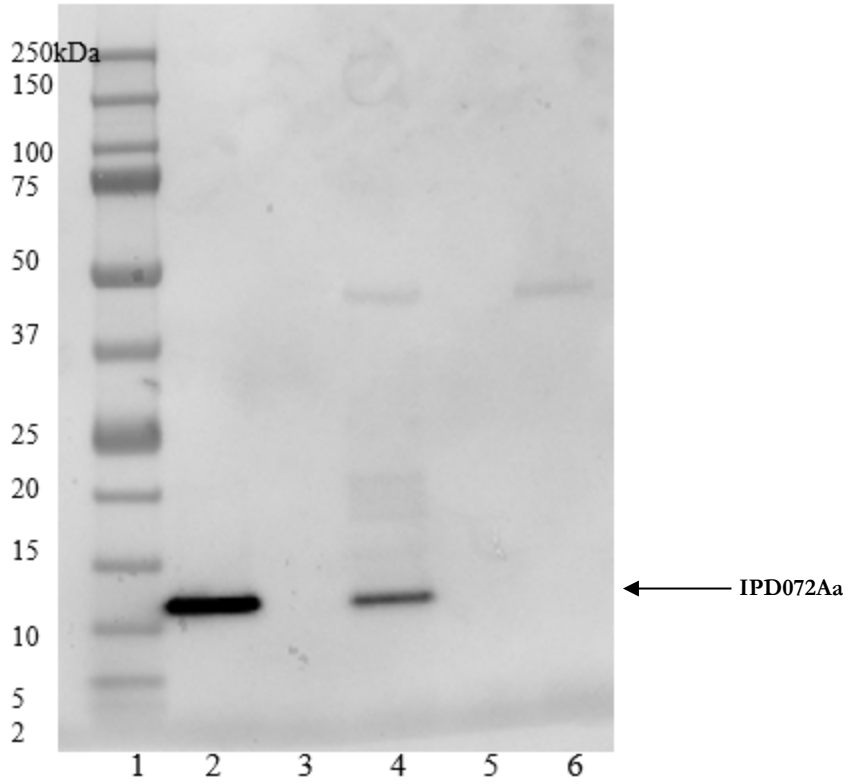
Figure 88. Dose Confirmation and Homogeneity Assessment of IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
6	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
8	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
9	IPD072Aa Protein Test Diet (Treatment 2; Week 2)
10	IPD072Aa Protein Test Diet (Treatment 2; Week 2)
11	IPD072Aa Protein Test Diet (Treatment 2; Week 2)
12	IPD072Aa Protein Test Diet (Treatment 2; Week 3)
13	IPD072Aa Protein Test Diet (Treatment 2; Week 3)
14	IPD072Aa Protein Test Diet (Treatment 2; Week 3)
15	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
16	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
17	IPD072Aa Protein Test Diet (Treatment 2; Week 4)

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of purified IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Day 0 and Weeks 1-4 refer to the approximate length of time for which samples were stored frozen (-20°C freezer unit) prior to analysis.

Figure 89. Frozen Storage Stability Assessment of IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	1X LDS/DTT Sample Buffer Blank
4	Heat-Treated Control Diet (Treatment 3; Day 0)
5	1X LDS/DTT Sample Buffer Blank
6	Bioassay Control Diet (Treatment 1; Day 0)

Note: Nanogram (ng) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Treatment 1 samples were loaded at the same matrix concentration as Treatment 2. Treatment 3 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2.

Figure 90. Assessment of the Presence or Absence of IPD072Aa Protein in Treatments 1 and 3

G5.a. Green Lacewing IPD072Aa Protein Bioassay Analytical Phase

The following dosing solutions were prepared for the Green Lacewing bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials (ASTM) Type 1) water

Test dosing solution used to prepare Treatment 2 consisting of test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 3 consisting of a portion of the test dosing solution used to prepare Treatment 2 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in the test diet (Treatment 2; 500 ng IPD072Aa protein per mg diet wet weight). The presence or absence of IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 3, respectively) and frozen storage stability of the IPD072Aa protein in Treatment 2 were also assessed.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation for the Green Lacewing bioassay. To generate the test dosing solution for Treatment 2, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (5.00 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 2 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the Green Lacewing bioassay, samples of Treatments 1, 2, and 3 were collected as shown in Table 206.

Table 206. Green Lacewing IPD072Aa Protein Bioassay Diet Samples

Treatment	Number of Samples	Analysis
2	5 beginning	Homogeneity of IPD072Aa protein
	5 middle	
	5 end	
2	NA (homogeneity samples were used)	Visual confirmation of IPD072Aa protein dose
2	30[a]	Frozen storage stability
1	1 each	Verify presence or absence of IPD072Aa protein
3		

Note: Not applicable (NA). Beginning, middle, and end refer to the stage of the diet aliquoting process at which samples were collected.

[a] Thirty sub-samples were placed in the freezer (-20 °C freezer unit). For each time point (Weeks 1-4), three were analyzed; homogeneity samples were used for Day 0 assessment and not frozen.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1, 2, and 3 were collected on dry ice into pre-weighed tubes. The tubes were re-weighed and samples were extracted targeting a ratio of 50 mg of sample extracted in 600 µl of extraction buffer (1X lithium dodecyl sulfate (LDS) sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing dithiothreitol, and 65% water). In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-20 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 5 minutes. Samples were then loaded into 4-12% Bis-Tris gels as as shown in Table 207.

Table 207. Green Lacewing IPD072Aa Protein Bioassay SDS-PAGE Samples

Treatment	Number of Samples	Analysis
2	3 beginning	Homogeneity
	3 middle	
	3 end	
2	2	Dose confirmation of IPD072Aa protein
2	3 per timepoint (Day 0, Weeks 1-4)	Frozen storage stability
1	1	Presence/absence of IPD072Aa protein
3	1	

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before and after the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for 60-90 minutes at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient temperature. The membranes were then washed with PBST four times for at least 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

G5.b. Green Lacewing IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 2 used in the Green Lacewing bioassay was evaluated by conducting a 7-day bioassay using WCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after completion of the Green Lacewing bioassay.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer.

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)

Treatment B: Test Diet (containing a portion of Treatment 2 and targeting 100 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 2 during diet preparation for the Green Lacewing bioassay were removed from the freezer (-20 °C freezer unit) and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with artificial diet for the WCR bioassay at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

For Treatment A, the resulting wet diet was mixed with Treatment 1 from the Green Lacewing bioassay, resulting in a 20% incorporation of the Green Lacewing diet by wet weight of the WCR diet.

For Treatment B, the resulting wet diet was mixed with Treatment 2 from the Green Lacewing bioassay, resulting in a 20% incorporation of the Green Lacewing diet by wet weight of the WCR diet.

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100, and are presented in Table 205. Weight data were summarized as means, standard deviations, and ranges in Table 205.

G6. Evaluation of the Survival and Development of Convergent Lady Beetle Fed Artificial Diets Containing IPD072Aa Protein

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0037-AP; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

Carrier

The carrier for the CNV bioassay consisted of *Ephestia* (*Ephestia kuehniella* sp.) eggs.

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure water.

The test dosing solutions used to prepare Treatments 2, 3, and 4 consisted of the test substance diluted in ultrapure water to achieve the concentration in each respective test diet.

The heat-treated control dosing solution used to prepare Treatment 5 consisted of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes as described in section G6.a. CNV IPD072Aa Protein Bioassay Analytical Phase.

The positive control dosing solution used to prepare Treatment 6 consisted of boric acid (H_3BO_3) and ultrapure water.

Test System

The test system was Hippodamia convergens (convergent lady beetle; Coleoptera: Coccinellidae). CNV was selected as a representative coleopteran to characterize the spectrum of activity of IPD072Aa protein. CNV larvae were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

The test system for the sensitive insect bioassay was Diabrotica virgifera virgifera (western corn rootworm; Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

CNV larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 100 ng IPD072Aa protein per mg diet dry weight)
- Treatment 3: Test Diet (targeting 500 ng IPD072Aa protein per mg diet dry weight)
- Treatment 4: Test Diet (targeting 1000 ng IPD072Aa protein per mg diet dry weight)
- Treatment 5: Heat-Treated Control Diet (targeting 1000 ng heat-treated IPD072Aa protein per mg diet dry weight)
- Treatment 6: Positive Control Diet (targeting 15,000 ng boric acid per mg diet dry weight)

Treatments were arranged in a randomized complete block design with a total of 30 blocks. Each block consisted of a stack of six Petri dishes secured together and contained one replicate from each treatment. Each treatment was fed to a target of 30 CNV individuals. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark. Larvae were refed every 3 to 4 days and assessed for pupation. Once pupation was observed, organisms were assessed daily for emergence and emerged adults were weighed. After 28 days, the bioassay was complete.

The bioassay acceptability criteria indicated the bioassay may be terminated and repeated if:

- The combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group
- The mortality of the positive control diet (Treatment 6) group does not exceed 80%

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in Treatments 2, 3, and 4 and the stability under bioassay conditions and the frozen storage stability of the IPD072Aa protein in Treatment 2. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 was also assessed. A sensitive insect bioassay was used to demonstrate the biological activity of IPD072Aa protein in Treatment 4 of the CNV bioassay.

Bias in the CNV bioassay and sensitive insect bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing.

Diet Generation

Each diet was prepared in bulk for the duration of the bioassay. Dosing solutions for Treatments 1-5 were prepared and maintained as described in the Analytical Phase section below. The positive control dosing solution for Treatment 6 was prepared by solubilizing and diluting boric acid in ultrapure water to achieve a nominal concentration of 15,000 ng/mg diet dry weight. Each dosing solution was mixed with carrier in a 0.75:1 ratio (i.e., 0.75 ml dosing solution to 1 g carrier) to generate Treatments 1-6. After mixing, diets were lyophilized and then pooled together by treatment. The bulk diets were aliquoted into individual storage tubes and stored frozen (-80 °C freezer unit) until use.

Sample Collection and Diet Characterization

During the process of diet aliquoting, samples of Treatments 1-5 were collected for characterization of diets as described in the Analytical Phase section below. In addition, a portion each of Treatment 1 and Treatment 4 was collected for use in diets (Treatments A and B, respectively) for the sensitive insect bioassay and stored frozen (-80 °C freezer unit). Preparation of diets for the sensitive insect bioassay is described in section G6.b. CNV IPD072Aa Protein Sensitive Insect Bioassay.

The positive control diet (Treatment 6) was not characterized.

CNV Bioassay

CNV eggs were incubated in an environmental chamber until the eggs hatched. CNV neonates were used in the bioassay within 24 hours of hatching.

On Day 0, diet aliquots were removed from storage and approximately 120 mg of diet were aliquoted into individual small caps. Caps containing the appropriate diet treatment and a moisture source (a tube filled with 0.5% agar) were distributed to each Petri dish utilized in the bioassay. One CNV neonate was placed in each dish and the dishes were stacked into blocks and secured together. The bioassay was conducted in an environmental chamber set at 27 °C, 65% relative humidity, and a photoperiod of 16 hours of light followed by 8 hours of dark for 28 days. Every 3-4 days, bioassay dishes were removed from the environmental chamber, pupation was assessed, and missing or dead organisms were recorded. For each living organism that had not yet pupated or died, old diet was removed from the dish and new diet-filled caps were prepared and distributed as described for Day 0, prior to returning the dishes to the environmental chamber. Once pupation was observed, organisms were assessed daily for adult emergence. CNV adults were weighed within approximately 24 hours of emergence. On Day 28, the bioassay was complete and any organisms that had not emerged were considered dead for the purpose of statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

The response variables of interest were mortality, weight and number of days to adult emergence. Statistical comparisons were made between CNV fed diet containing IPD072Aa (Treatment 2, 3, or 4) and the bioassay control diet (Treatment 1) for each response variable.

Mortality

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of CNV fed each of the artificial insect diets containing IPD072Aa protein (m_T) was greater than the mortality rate of those fed the bioassay control diet (m_C). The corresponding hypothesis tests were

$$H_0 : m_T - m_C = 0 \quad vs. \quad H_a : m_T - m_C > 0 .$$

A significant difference was identified if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Weight

A linear mixed model analysis was conducted to test if exposure to IPD072Aa protein caused growth inhibition. Weight was treated as the response and analyzed using the model:

$$y_{ij} = W_i + \varepsilon_{ij}$$

where W_i denotes the mean of the i^{th} treatment (fixed effect) and ε_{ij} denotes the error term associated with the observation obtained from the j^{th} sample of the i^{th} treatment. For this model, it was assumed that errors $\varepsilon_{ij} \sim N(0, \sigma^2_\varepsilon)$ were independently and identically distributed. This assumption was confirmed by inspection of the residuals from the fitted model.

Each diet containing IPD072Aa protein (w_T) was compared to the bioassay control diet (w_C) to determine if the weight of *CNV* fed the test diet was reduced. The corresponding hypothesis tests were

$$H_0 : w_T - w_C = 0 \quad vs. \quad H_a : w_T - w_C < 0$$

A significant difference was identified if the P-value was < 0.05. SAS PROC GLIMMIX was utilized for linear mixed model analysis and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Number of Days to Adult Emergence

The distributions of days to adult emergence were evaluated for each treatment. The data were not normally distributed; therefore, non-parametric two-sample tests were conducted separately to examine if exposure to IPD072Aa protein of each concentration caused developmental delays compared to exposure to the bioassay control diet. The corresponding hypothesis tests were

$$H_0 : F_T(x) = F_c(x) \ \ vs. \ H_a : F_T(x) > F_c(x)$$

Where $F_T(x)$ is the cumulative distribution of the number of days to adult emergence of *CNV* fed the artificial diet containing IPD072Aa protein, and $F_c(x)$is the cumulative distribution of the number of days to adult emergence of individuals fed the bioassay control diet. Therefore, significance would indicate that insects fed the diet containing IPD072Aa protein would have a greater probability to take longer to emerge than those fed the control diet.

The Wilcoxon two-sample test was conducted to evaluate the above hypothesis. The Siegel-Tukey test was conducted to further test for differences in scale between the two treatments, as the Wilcoxon test is not effective for evaluating scale differences (Gibbons and Chakraborti, 1992). The Siegel-Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY. A significant difference was established if the P-value was < 0.05.

Demonstration of IPD072Aa Protein Activity in the *CNV* Test Diet

A sensitive insect bioassay was performed using *WCR* larvae to demonstrate the biological activity of the IPD072Aa protein used in Treatment 4 in the *CNV* bioassay. Details regarding the sensitive insect bioassay are provided in Appendix B.

Results and Discussion

The CNV bioassay met the acceptability criteria as the combined dead and missing organism count in the bioassay control diet (Treatment 1) group did not exceed 20% and mortality in the positive control diet (Treatment 6) group exceeded 80%, as shown in Table 208.

The mortality of CNV fed the test diets containing 100 ng/mg and 500 ng/mg IPD072Aa protein (Treatments 2 and 3, respectively; 0%; P-value = 1.0000) was not significantly greater than those fed the bioassay control diet (Treatment 1; 0%), as shown in Table 208. The mortality of CNV fed the test diet containing 1000 ng/mg IPD072Aa protein (Treatment 4; 56.7%; P-value < 0.0001) was significantly greater than those fed the bioassay control diet (Treatment 1), as shown inTable 208.

The mean weight of newly emerged adult CNV fed Treatments 2, 3, and 4 (18.6 mg, 11.3 mg, and 8.63 mg, P-values = 0.0236, < 0.0001, and < 0.0001, respectively) was significantly less than those fed Treatment 1 (19.7 mg), as shown in Table 209 While there was an observed statistical difference between CNV fed 100 ng/mg IPD072Aa and the control diet, this result is not thought to be biologically significant. Rodriguez-Saona and Miller (1999) showed similar levels of variability in CNV adult weight in experiments assessing maturation differences when reared under four different temperatures. Thus, it is unlikely that an approximately 1 mg difference in mean adult weight is biologically relevant in this context.

CNV fed Treatment 2 (median 14 days; Wilcoxon test P-value 0.0551) and Treatment 1 (median 14 days) did not significantly differ in their probability to take longer to emerge (measured as days to adult emergence) as shown in Table 210. In addition, the Siegel-Tukey test (P-value 0.7594) did not show significant evidence that the scales of the two populations differed. However, Treatments 3 and 4 (median 17 and 22 days, respectively; Wilcoxon test P-values 0.0001, each) both had significantly greater probability to take longer to emerge than those fed Treatment 1. In addition, the Siegel-Tukey test for Treatment 3 (P-value 1.0000) did not show significant evidence that the scales of the populations differed. For Treatment 4, the Siegel-Tukey test (P-value 0.0015) did show significant evidence that the scales of the populations differed.

Western blot analysis visually confirmed the dose and homogeneity of the IPD072Aa protein in Treatments 2-4 (Figure 91; Figure 92; Figure 93; Figure 94. The stability under bioassay conditions of the IPD072Aa protein in Treatment 2; Figure 95), and frozen storage stability of Treatment 2 (Figure 6), were also assessed. The presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5 were also assessed (Figure 91). The IPD072Aa protein was detected in the heat-treated control diet (Treatment 5; Figure 91); however, the band was less intense than that of the test diets (Treatments 2, 3, and 4). No IPD072Aa protein band was detected in the bioassay control diet (Treatment 1; Figure 91).

Observed larval mortality and weight data for the WCR sensitive insect bioassay are summarized in Table 211. The WCR bioassay met the acceptability criterion (Sensitive Insect Bioassay section below). The biological activity of the IPD072Aa protein in Treatment 4 of the CNV bioassay was demonstrated by increased mortality and decreased weight of WCR fed the test diet (Treatment B; described in the Sensitive Insect Bioassay section below).

Conclusion

The results demonstrated the mortality for CNV fed the test diets containing 100 ng and 500 ng IPD072Aa protein per mg diet (Treatment 2 and Treatment 3, respectively; both 0%) was not significantly greater than the mortality for CNV fed the bioassay control diet (Treatment 1;

0.0%). A statistically significant difference was observed in mortality between CNV fed the test diet containing 1000 ng IPD072Aa protein per mg diet (Treatment 4; 56.7%) and those fed Treatment 1. Statistically significant differences were observed in mean weight between CNV fed Treatments 2, 3 and 4 (18.6 mg, 11.3 mg, and 8.63 mg, respectively) and those fed Treatment 1 (19.7 mg). CNV fed Treatment 2 (median 14 days) and Treatment 1 (median 14 days) did not significantly differ in their probability to take longer to emerge. CNV fed Treatments 3 and 4 (median 17 and 22 days, respectively) had significantly greater probability to take longer to emerge than those fed Treatment 1.

Table 208. Summary Analysis of CNV IPD072Aa Protein Bioassay Mortality Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)	Fisher's Test P-Value
1	Bioassay Control Diet	0	30	0	0	--
2	Test Diet	100	30	0	0	1.0000
3	Test Diet	500	30	0	0	1.0000
4	Test Diet	1000	30	17[a]	56.7	<0.0001[b]
5	Heat-treated Control Diet	1000	30	1	3.33	--
6	Positive Control Diet	0[c]	30	30	100	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 were based on diet dry weight.

[a] Two organisms had not emerged as adults by Day 28 of the bioassay and were scored as dead.

[b] A statistically significant difference (P-value < 0.05) was observed.

[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 209. Summary Analysis of CNV IPD072Aa Protein Bioassay Adult Weight Results

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean (95% Confidence Interval) (mg)	Range (mg)	P-Value
1	Bioassay Control Diet	0	30	19.7 (18.9 - 20.4)	16.6 - 23.8	--
2	Test Diet	100	30	18.6 (17.9 - 19.3)	11.6 - 22.6	0.0236[a]
3	Test Diet	500	30	11.3 (10.6 - 12.1)	8.9 - 15.2	<0.0001[a]
4	Test Diet	1000	13	8.63 (7.51 - 9.75)	6.9 - 11.7	<0.0001[a]
5	Heat-treated Control Diet	1000	29	18.5 ± 3.63[b]	11.5 - 25.1	--
6	Positive Control Diet	0[c]	0	NA	NA	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving *Hippodamia convergens* in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

[b] Standard deviation is provided for mean values not subjected to a *t*-test.

[c] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 210. Summary Analysis of CNV IPD072Aa Protein Bioassay Results for Days to Adult Emergence

Treatment	Treatment Description	Treatment Dose (ng IPD072Aa/mg)	Number of Surviving Organisms	Mean± Standard Deviation (days)	Median (days)	Range (days)	Wilcoxon Test P-Value	Siegel-Tukey Test P-Value
1	Bioassay Control Diet	0	30	13.8 ± 0.664	14	13 - 15	--	--
2	Test Diet	100	30	14.1 ± 0.712	14	13 - 16	0.0551	0.7594
3	Test Diet	500	30	17.4 ± 1.63	17	15 - 21	<0.0001[a]	1.0000
4	Test Diet	1000	13	22.3 ± 2.25	22	19 - 25	<0.0001[a]	0.0015[a]
5	Heat-treated Control Diet	1000	29	14.6 ± 1.50	14	13 - 20	--	--
6	Positive Control Diet	0[b]	0	NA	NA	NA	--	--

Note: Targeted IPD072Aa protein concentrations in Treatments 2-5 are based on diet dry weight. Not applicable (NA); there were no surviving *Hippodamia convergens* in Treatment 6.

[a] A statistically significant difference (P-value < 0.05) was observed.

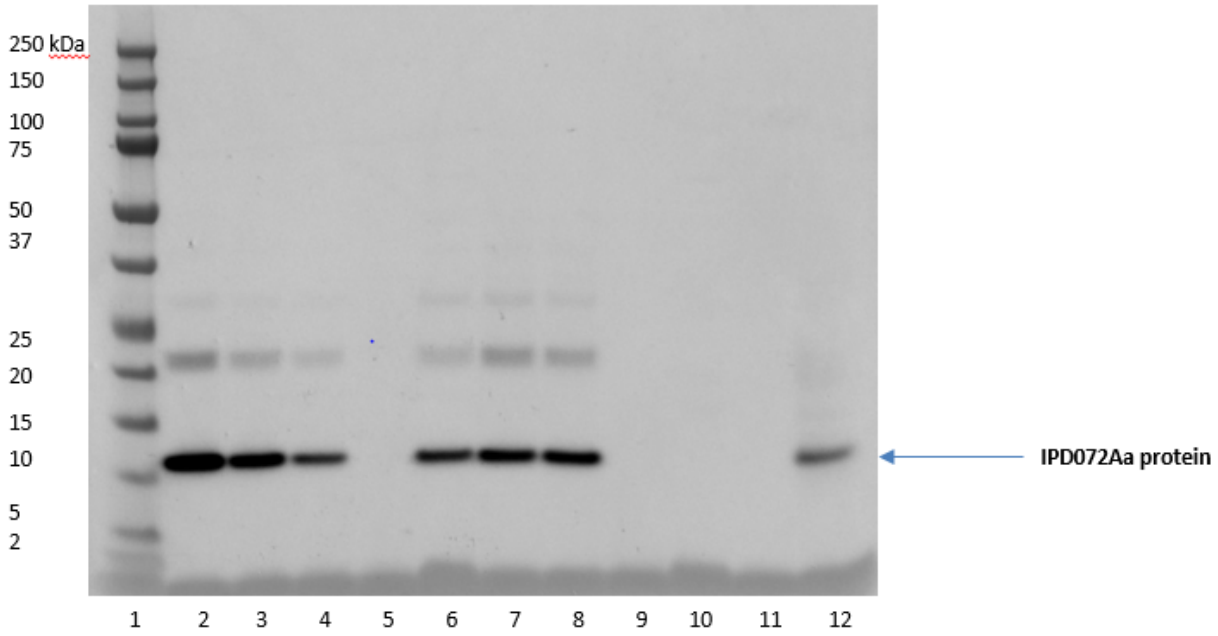
[b] Treatment 6 contained a targeted concentration of 15,000 ng boric acid per mg diet dry weight.

Table 211. Summary of CNV IPD072Aa Protein Sensitive Insect Bioassay Results

Treatment	Treatment Description	Treatment Dose	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0 ng IPD072Aa/mg	29[a]	6.90	27	0.415 ± 0.149	0.1 - 0.7
B	Test Diet	85.5 ng IPD072Aa/mg	26[a]	92.3	2	0.300 ± 0.283	0.1 - 0.5

Note: Treatments A and B used in the sensitive insect (*Diabrotica virgifera virgifera*) bioassay were prepared from the same bulk diet preparations used in Treatments 1 and 4, respectively, of the *Hippodamia convergens* bioassay. The concentration of IPD072Aa protein in Treatment B was based on the wet weight of the artificial diet.

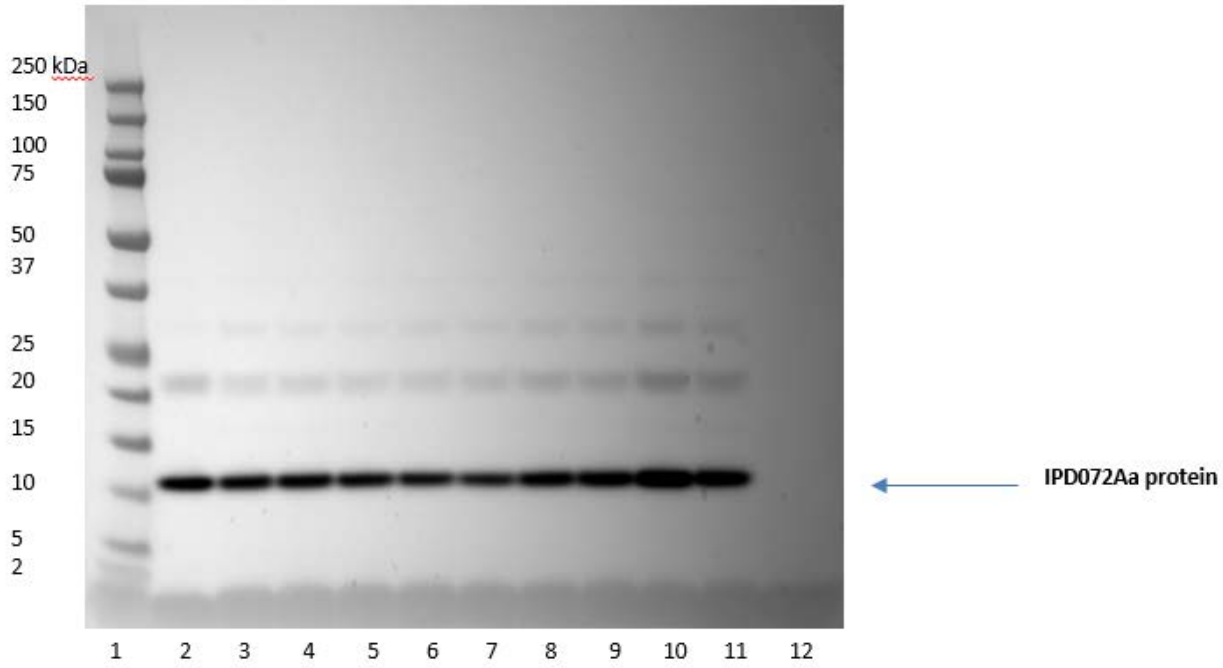
[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (20 ng)
3	Test Substance (10 ng)
4	Test Substance (5 ng)
5	1X LDS Sample Buffer Blank
6	IPD072Aa Protein Test Diet (Treatment 2)
7	IPD072Aa Protein Test Diet (Treatment 3)
8	IPD072Aa Protein Test Diet (Treatment 4)
9	1X LDS Sample Buffer Blank
10	Bioassay Control Diet (Treatment 1)
11	1X LDS Sample Buffer Blank
12	Heat-Treated Control Diet (Treatment 5)

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Treatment 1 was loaded diluted to the same matrix concentration as Treatment 2. Treatment 2, 3, and 4 were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in each respective treatment. Treatment 5 samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

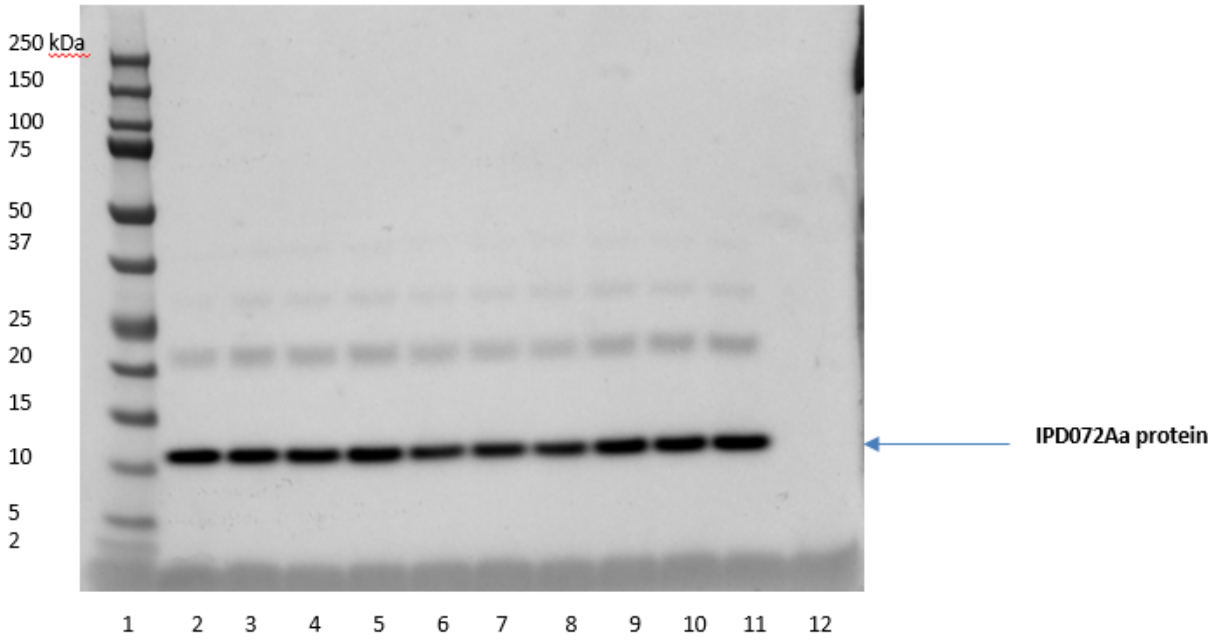
Figure 91. Dose Confirmation of the IPD072Aa Protein in Treatments 2, 3, and 4 and Assessment of the Presence or Absence of Immunodetectable IPD072Aa Protein in Treatments 1 and 5



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 2; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 2; Middle)
7	IPD072Aa Protein Test Diet (Treatment 2; Middle)
8	IPD072Aa Protein Test Diet (Treatment 2; Middle)
9	IPD072Aa Protein Test Diet (Treatment 2; End)
10	IPD072Aa Protein Test Diet (Treatment 2; End)
11	IPD072Aa Protein Test Diet (Treatment 2; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Beginning, middle, and end refer to the stages in the diet aliquoting process at which the samples were collected.

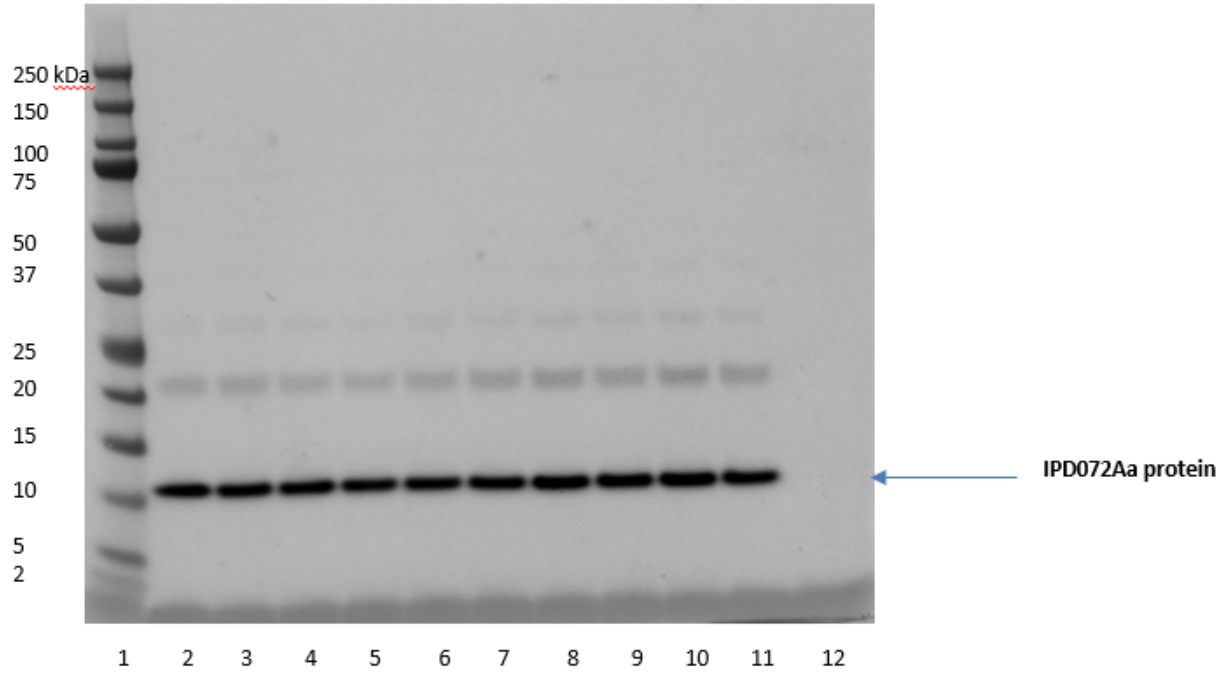
Figure 92. Homogeneity Assessment of the IPD072Aa Protein in Treatment 2



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 3; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 3; Middle)
7	IPD072Aa Protein Test Diet (Treatment 3; Middle)
8	IPD072Aa Protein Test Diet (Treatment 3; Middle)
9	IPD072Aa Protein Test Diet (Treatment 3; End)
10	IPD072Aa Protein Test Diet (Treatment 3; End)
11	IPD072Aa Protein Test Diet (Treatment 3; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein Treatment 3.

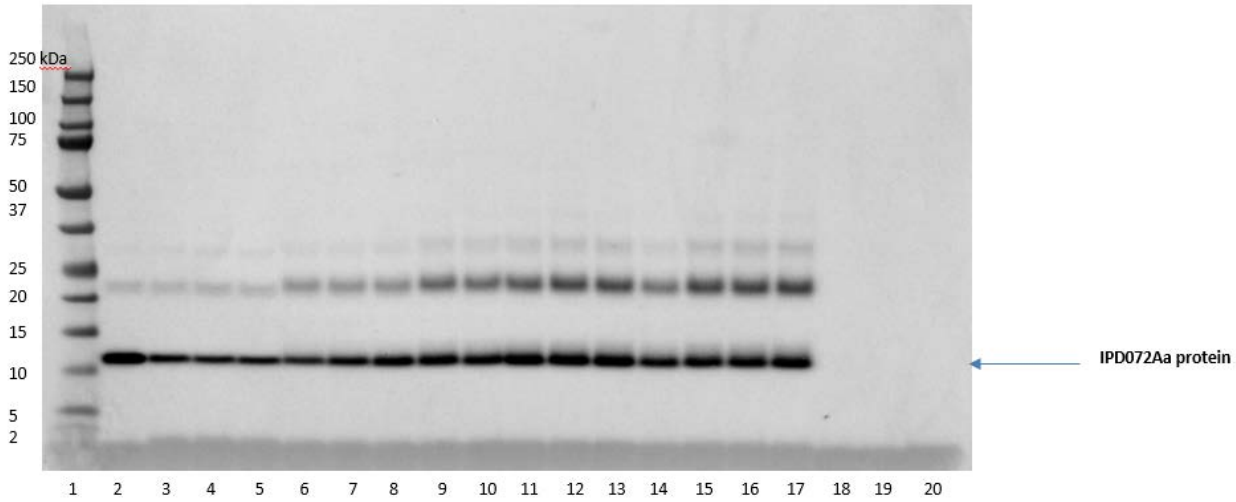
Figure 93. Homogeneity Assessment of the IPD072Aa Protein in Treatment 3



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
4	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
5	IPD072Aa Protein Test Diet (Treatment 4; Beginning)
6	IPD072Aa Protein Test Diet (Treatment 4; Middle)
7	IPD072Aa Protein Test Diet (Treatment 4; Middle)
8	IPD072Aa Protein Test Diet (Treatment 4; Middle)
9	IPD072Aa Protein Test Diet (Treatment 4; End)
10	IPD072Aa Protein Test Diet (Treatment 4; End)
11	IPD072Aa Protein Test Diet (Treatment 4; End)
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 4.

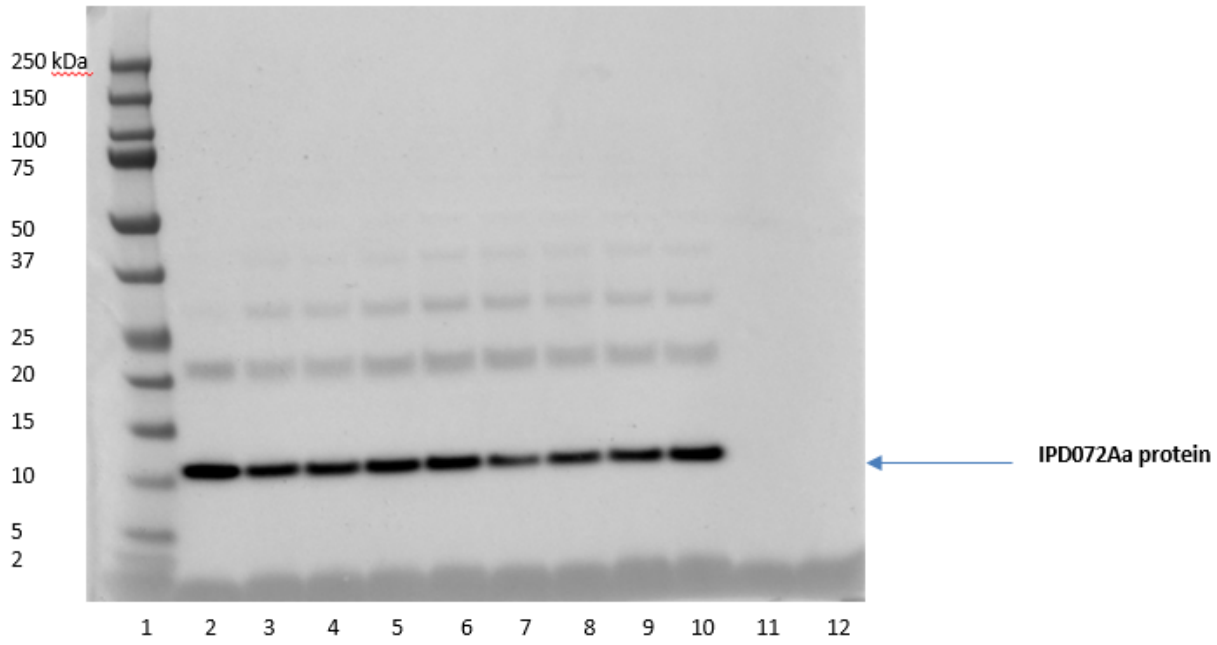
Figure 94. Homogeneity Assessment of the IPD072Aa Protein in Treatment 4



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	IPD072Aa Protein Test Diet (Treatment 2; Day 2)
2	Test Substance (10 ng)	12	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	13	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	14	IPD072Aa Protein Test Diet (Treatment 2; Day 3)
5	IPD072Aa Protein Test Diet (Treatment 2; Day 0)	15	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
6	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	16	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
7	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	17	IPD072Aa Protein Test Diet (Treatment 2; Day 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Day 1)	18	1X LDS Sample Buffer Blank
9	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	19	1X LDS Sample Buffer Blank
10	IPD072Aa Protein Test Diet (Treatment 2; Day 2)	20	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Days 0-4 refer to the number of days for which samples were stored under bioassay conditions prior to analysis.

Figure 95. Stability Assessment of the IPD072Aa Protein in Treatment 2 under Bioassay Conditions



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers
2	Test Substance (10 ng)
3	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
4	IPD072Aa Protein Test Diet (Treatment 2; Day 0)
5	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
6	IPD072Aa Protein Test Diet (Treatment 2; Week 1)
7	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
8	IPD072Aa Protein Test Diet (Treatment 2; Week 4)
9	IPD072Aa Protein Test Diet (Treatment 2; Week 6)
10	IPD072Aa Protein Test Diet (Treatment 2; Week 6)
11	1X LDS Sample Buffer Blank
12	1X LDS Sample Buffer Blank

Note: Nanogram (ng), lithium dodecyl sulfate (LDS). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix. The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in Treatment 2. Week 1, Week 4, and Week 6 refer to the approximate length of time samples were stored frozen (-80°C freezer unit) prior to analysis.

Figure 96. Frozen Storage Stability Assessment of the IPD072Aa Protein in Treatment 2

G6.a. CNV IPD072Aa Protein Bioassay Analytical Phase

The following IPD072Aa protein dosing solutions and control dosing solutions were prepared for the CNV bioassay:

Bioassay control dosing solution used to prepare Treatment 1 consisting of ultrapure (American Society for Testing and Materials ASTM Type 1) water

Test dosing solutions used to prepare Treatment 2, 3, and 4 consisting of IPD072Aa protein test substance diluted in ultrapure water to achieve the concentrations in the test diets

Heat-treated control dosing solution used to prepare Treatment 5 consisting of a portion of the test dosing solution used to prepare Treatment 4 that had been autoclaved (121 °C, 20 psi) for 30 minutes

Western blot analysis was used to visually confirm the dose and homogeneity of the IPD072Aa protein in three test diets (Treatments 2, 3, and 4; 100 ng, 500 ng, and 1000 ng IPD072Aa protein per mg diet dry weight, respectively). The presence or absence of immunodetectable IPD072Aa protein in the bioassay control and heat-treated control diets (Treatments 1 and 5, respectively), stability under bioassay conditions and frozen storage stability of the IPD072Aa protein in Treatment 2 were also assessed.

Preparation of Solutions

Dosing solutions were prepared on the day of diet preparation. To generate the test dosing solutions for Treatments 2, 3, and 4, aliquots of the test substance were thawed under chilled conditions, pooled together, and then diluted in ultrapure water to the appropriate IPD072Aa protein concentration (0.133 mg/ml, 0.667 mg/ml, and 1.333 mg/ml, respectively). To generate the heat-treated IPD072Aa protein control dosing solution, a portion of the test dosing solution used to prepare Treatment 4 was autoclaved for 30 minutes at a setting of 121 °C and 20 psi to inactivate the IPD072Aa protein. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were maintained chilled until use.

Characterization of Diets

Sample Collection

During the process of diet aliquoting for the CNV bioassay, samples were collected as shown in Table 212.

Table 212. CNV IPD072Aa Protein Bioassay Diet Samples

Treatment	Number of Samples	Analysis
2	5 beginning	Homogeneity of IPD072Aa protein
	5 middle	
	5 end	
3	5 beginning	
	5 middle	
	5 end	
4	5 beginning	
	5 middle	
	5 end	
2	NA (One homogeneity sample was used from each treatment)	Visual confirmation of IPD072Aa protein dose
3		
4		
1	1 from each treatment	Verify presence or absence of immunodetectable IPD072Aa protein
5		
1	5 (for Day 0 assessment)	Oven dry weight equivalence
	20 (distributed to dishes)	
2	NA (3 homogeneity samples were used for Day 0 assessment)	Stability under bioassay conditions
	20 (distributed to Petri dishes)	
2	NA (2 homogeneity samples were used for Day 0 assessment)	Frozen storage stability
	2 (for each timepoint; Week 1, Week 4, and Week 6)	

Stability under Bioassay Conditions and Determination of Oven Dry Weight Equivalence

For assessment of stability under bioassay conditions, the aliquots of Treatment 1 and Treatment 2 were removed from storage, distributed to dishes (20 for each treatment), and placed under bioassay conditions using the same methods and conditions used in the *CNV* bioassay, except they were not infested with larvae. Day 0 samples for Treatment 1 and Treatment 2 were not placed under bioassay conditions.

For each day of stability analysis (Days 1-4), five samples each of Treatment 1 and Treatment 2 were removed from bioassay conditions. Treatment 2 samples were prepared for SDS-PAGE analysis and Treatment 1 samples were used to calculate oven dry weight equivalence.

As stability under bioassay conditions is assessed on a dry weight basis, the average oven dry weight equivalence (ODE) of five Treatment 1 samples for each sampling time point (Days 0-4) was calculated. Treatment 1 ODE samples were placed into pre-weighed drying containers. A combined weight of wet sample and drying container was obtained for each sample and then samples were placed in an oven set at 100 °C for at least 18 hours.

Containers with dried samples were re-weighed and the ODE was calculated for each sample. The average ODE for each sampling time point was used to determine the extraction buffer volume needed for Treatment 2 bioassay stability samples.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sub-samples of Treatments 1, 2, 3, 4, and 5 were weighed to approximately 10 mg on dry ice in preparation for SDS-PAGE. For dose confirmation and assessment of homogeneity in Treatments 2, 3, and 4, Day 0 stability under bioassay conditions and storage stability in Treatment 2, and the presence or absence of immunodetectable IPD072Aa protein in Treatments 1 and 5, sub-samples were extracted in 600 µl of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water). For assessment of stability under bioassay conditions in Treatment 2 (Days 1-4), sub-samples were extracted in 1X LDS sample buffer with volumes normalized depending on the average ODE for each time point. In addition, undiluted sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples were thawed and diluted, as applicable, either before or after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels as shown in Table 213.

Table 213. CNV IPD072Aa Protein Bioassay SDS-PAGE Samples

Gel	Treatment	Number of Samples
Dose confirmation of IPD072Aa protein	2, 3, and 4	1 per treatment
Homogeneity	2	3 beginning
		3 middle
		3 end
	3	3 beginning
		3 middle
		3 end
	4	3 beginning
		3 middle
		3 end
Stability under bioassay conditions	2	3 per timepoint (Days 0-4)
Storage Stability	2	2 per timepoint (Day 0, Weeks 1, 4, and 6)
Presence/absence of IPD072Aa protein	1	1
	5	1

One or more dilutions of the test substance were also loaded into each gel along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for approximately 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for at least 1 minute each to reduce the background. The blocked membranes were incubated with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for at least 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for approximately 1 hour at ambient temperature. The membranes were then washed with PBST four times for at least 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa. Bands that may be visible at other molecular weights can be attributed to oligomer formation in the lanes that include test substance and/or non-specific binding to the diet matrix.

G6.b. CNV IPD072Aa Protein Sensitive Insect Bioassay

The biological activity of the IPD072Aa protein in Treatment 4 used in the CNV bioassay was evaluated by conducting a 7-day bioassay usingWCR, a species sensitive to IPD072Aa protein. The WCR bioassay was initiated after the CNV bioassay was complete.

WCR (western corn rootworm; Coleoptera: Chrysomelidae) eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel. The carrier for the WCR bioassay consisted of an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

WCR larvae were exposed via oral ingestion to one of the following two treatments:

Treatment A: Bioassay Control Diet (containing a portion of Treatment 1)

Treatment B: Test Diet (containing a portion of Treatment 4 and targeting 85.5 ng IPD072Aa protein per mg WCR diet wet weight)

Treatments were arranged in a generalized randomized block design with a total of three blocks. Each block consisted of a 24-well bioassay plate and contained 10 replicates from each treatment. Each treatment was fed to a target of 30 WCR individuals.

Diet Preparation

Stored aliquots collected from Treatments 1 and 4 during diet preparation for the CNV bioassay were removed from the freezer (-80 °C freezer unit) and used to prepare Treatments A and B (Day 0 and Day 4 diet preparation) for the WCR bioassay as follows:

For Treatment A, artificial diet for the WCR bioassay was mixed with Treatment 1 from the CNV bioassay, resulting in a 30% incorporation of the CNV diet by dry weight of the WCR diet.

For Treatment B, artificial diet for the WCR bioassay was mixed with Treatment 4 from the CNV bioassay, resulting in a 30% incorporation of the CNV diet by dry weight of the WCR diet.

For each respective treatment, ultrapure (American Society for Testing and Materials ASTM Type 1) water was mixed with the dry ingredients at a 2.51:1 ratio (i.e., 2.51 ml water to 1 g carrier).

WCR Bioassay

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each

bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and a 24-hour dark cycle for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

The bioassay acceptability criteria indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment A) group.

Data Analysis

Mortality data for the sensitive insect bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100 and are presented in Table 211 Weight data were summarized as means, standard deviations, and ranges and are presented in Table 211.

G7. Northern Bobwhite (*Colinus virginianus*) Acute Oral Toxicity Limit Test with Recombinant IPD072Aa Protein

Materials and Methods

Study Protocol

The methods described in this protocol meet the testing requirements of the OCSPP Guideline 850.2100 (US-EPA, 2012).

Test Substance

The test substance, IPD072Aa, Lot Number PCF-0040, was received on 3 August 2017 from Pioneer Hi-Bred International, Inc., Johnston, Iowa, USA (Pioneer). The sample was tested as whole product and was not corrected for the purity of a specific active ingredient. Upon receipt at the CRO, the test substance was given a unique Test Material Center (TMC) number (No. 18-44), and was stored frozen at <-60^{o} C in the original container in freezer F-32.

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substance are the responsibility of Pioneer. A retention sample (approximately 25 mg) of the test and comparative control substances was collected and stored frozen (< −20^{o}C). This retention sample will be retained for a period not to exceed 12 months from time of collection. All unused bulk test substance, except the retention sample, was shipped on dry ice via overnight courier, to Pioneer after completion of the in-life phase of the study:

Test Organism

The northern bobwhite (*Colinus virginianus*) was selected as the test organism since it is recommended by the U.S. EPA, and is commonly used, as the model of upland gamebirds in toxicity tests of pesticides. A substantial database exists on the response of this species to pesticides. Oral exposure to pesticides is considered appropriate because free-ranging birds may ingest pesticides along with their food items. The northern bobwhites used during this study were obtained from The CRO's in-house quail colony number 17-A-23. Birds used in this study were phenotypically indistinguishable from wild stock, all from the same hatch, and were young adults approaching their first breeding season. Of those received, 20 individuals (10 males and 10 females) were randomly selected for use in the study.

The test animals were acclimated to individual test cages and study room conditions for two weeks prior to testing. The northern bobwhites used in this study were 16 weeks of age and weighed between 165.3 g and 196.9 g at experimental start. One male bird in the colony was euthanized during acclimation after it was found in its cage with a broken leg, which was assumed to be the result of a cage injury. There was no other mortality in the colony prior to

the experimental start date and the test animals demonstrated no signs of illness or disease before the initiation of the test.

Test Conditions

Housing

Test cage dimensions were 53 x 25.2 x 20.5 to 25 cm. The cages were made of epoxy-coated wire mesh bottoms, fronts, backs and tops with solid galvanized metal partitions. A solid catch pan was placed beneath each cage, and was lined with absorbent paper. Cages were made of non-absorbent materials and elevated to allow feces to pass through the cage floor, thereby minimizing contact with excreta and to minimize disease potential. Feed was provided by aluminum mini loaf pans. Water was provided by automatic drip waterers with cups. The system was plumbed into the facility's water supply.

Environmental Conditions

Room conditions were maintained according to the CRO SOP. Ventilation within the study room was provided at 51.7 room exchanges per hour. Daily minimum and maximum temperatures and relative humidity ranges within the test room were monitored daily. During acclimation, study room conditions were maintained at 19º C to 27º C and 56% to 70% relative humidity. During the definitive test, study room conditions were maintained at 18º C to 25º C and 49% to 69% relative humidity. Temperature and humidity were monitored using a digital minimum/maximum thermometer/hygrometer with memory.

Lighting within the test room was provided by LED bulbs. The light schedule was set at 10 hours light: 14 hours dark, with two 15-minute transition periods, one in the morning and one in the evening. Light intensity averaged 39.9 foot-candles during this study. Light intensity was measured with digital light meter LM03.

Feed and Water

Purina game bird flight conditioner was provided as the diet during the acclimation and experimental phases of the study. This diet is nutritionally sufficient to maintain gamebirds and has supported healthy control birds in numerous past acute studies conducted at the testing facility. A representative sample from the lot of feed used during this study was analyzed for the presence of pesticides, PCBs and toxic metals by Columbia Food Laboratories, Inc., Corbett, Oregon, using ISO/IEC 17025:2005 standards. None of the pesticides tested in the contaminant screen were detected at concentrations that are considered toxic to northern bobwhite in any of the samples analyzed. Therefore, the food source was considered as acceptable in quality.

Water was supplied from a well located at the test facility. Representative samples of the water are tested for the presence of pesticides, PCBs and toxic metals by Columbia Food Laboratories, Inc., Corbett, Oregon, using ISO/IEC 17025:2005 standards. None of these compounds were detected at concentrations considered toxic in any of the samples analyzed. Feed and water were provided to the test organisms *ad libitum.*

Randomization and Control of Bias

Test cages were numbered and randomized using the random number generator in Microsoft® EXCEL. During cage assignment, individual birds were indiscriminately selected from the colony, assigned a unique identification number using Monel #1005-3 numbered aluminum wing tags, and placed in test cages following the randomized list until 14 males and 14 females were assigned to test cages. Prior to test initiation, suitable birds were selected from those acclimated, then randomly assigned to test group according to cage number such that 5 males and 5 females were designated for each of the two test groups in this limit test. A total of 20 birds were used in the study.

Dose Preparation and Administration

Dose Levels

Relative to the test substance, the dose levels were 0 and 2,000 mg a.i./kg body weight.

Control substance: The Bovine Serum Albumin (BSA) used as the control substance was 100% pure and individual doses were prepared at 2,000 mg BSA/kg body weight for each control bird.

Test substance: The test substance dose level was 2,000 mg IPD072 Aa protein/ kg bird body weight. The lyophilized powder contained 0.80 mg IPD072Aa protein per mg powder. Therefore, the dose was corrected for 80% concentration to assure maximum exposure to the IPD072Aa protein (Kg body weight x 2,000 mg / 0.80).

Dose Preparation and Administration

Acclimated birds were fasted for at least 15 hours, during darkness, overnight between Day -2 and Day -1. They were weighed, and the weights recorded, the morning of 29 August 2017 (Day -1). Feed was returned to the birds as soon as all weights were recorded. The fasted weights were used to select birds with body weights within 15% of the mean of the test population, and that had approximately equal variance among groups for use in the study and to calculate individual doses. The volume of lyophilized protein test substance (TS) necessary to achieve 2000 mg/kg body-weight dose for each bird in the treatment group was greater than could be contained into a single dosing tube. Therefore, two dosing tubes were prepared for each bird. The individually calculated doses were weighed out on an analytical scale and placed into tared aluminum weigh boats. The TS was transferred from the weigh boats into two dosing tubes,

with approximately equal portions of the total dose. Each tube was labelled with the cage number of the bird for which the dose was prepared. The dose tubes were prepared in the evening of August 29 (completed about 8 PM), and placed in a metal pan, with aluminum foil cover to assure darkness, and maintained at room temperature until used for dosing the following morning (30 August 2017).

The volume of the comparative control substance (BSA) necessary for the individual control bird doses was easily contained in a single dosing tube. However, because the treatment group birds would be dosed using two dosing tubes, a second placebo tube was prepared for each control group bird and marked and stored as described above so that each bird would experience two dosing tube insertion procedures.

The birds were again fasted for 15 hours during overnight darkness the night prior to initiation of dosing. Each bird was removed from its respective cage by the assigned bird handler and presented, in the appropriate position, to the individual performing the dosing.

The first of two dosing tubes was inserted into each bird's crop. The dosing tube plunger was then pressed into the dosing tube, ejecting the dosing tube contents into the crop of the bird. All birds were dosed twice (with two different dosing tubes) in this manner. The control-group dosing process was conducted first, starting with the BSA in the first dosing tube followed by the placebo second dosing tube. Then the treatment-group dosing process was conducted. After each dose, a small amount of water (approximately 0.2 ml) was injected into the crop using a ball-tipped gavage needle on a 1 ml syringe. The intent of the water was to dampen the very light powder material in the crop to prevent regurgitation and potential subsequent inhalation into the trachea. Treatment bird 1978 (cage 1) ejected a small flake of test substance immediately after dosing and prior to administration of the water droplet. The flake was examined and determined to be substantially less than 1% of the dose. No further regurgitation/ejection was observed, indicating the water step was effective. The exact dose prepared for each bird is provided in Table 1. Feed was returned to the test system immediately after all birds were dosed.

Test Monitoring

The birds were dosed at "time 0" (experimental start). Total test duration was 14 days, measured in fourteen 24-hour intervals.

Behavioral Observations

Regurgitation, mortality, general condition, overt signs of toxicity and abnormal behavior were monitored within the test system. Each animal was carefully observed for 60 consecutive minutes post-dosing, then at three additional time points during the remaining photoperiod on Day 0. Observations were conducted twice daily during Days 1 through 10, and once daily, thereafter, until the end of the test (14 days).

Post-Mortem Examinations

There were no test animal fatalities during the study. Post-mortem examinations of gross pathology were conducted on all birds in the study at the end of the 14-day study period. The post-mortem examinations included evaluation of general physical condition, digestive tract, liver, kidneys, lungs, gall bladder, breast muscles, heart, and spleen.

Body Weight Determination

Fasted body weights were measured within 24 hours prior to dosing. Body weights were again measured for each test animal at 7 and 14 days (study termination) post-dosing. Measured body weights were used to calculate change in body weight from Day -1 to Day 7, Day 7 to Day 14, and Day -1 to Day 14 for each animal. Body weight change was calculated as follows, using the Day -1 to Day 7 interval as an example:

(measured body weight Day 7) – (measured body weight Day -1)

Feed Consumption

Feed consumption was measured during the experimental phase for each cage during the following intervals: Day 0 to Day 7 and Day 7 to Day 14. Spillage was prevented by the design of the feed cups used. No spillage was recorded. The total feed consumption per bird was calculated for each period and divided by the number of days the bird was alive to calculate daily feed consumption per bird. Food consumption per bird per day was calculated as follows, using the Day 7 to Day 14 interval as an example:

$$\frac{\text{(grams food provided Day 7) – (grams food remaining Day 14)}}{\text{\# days bird was alive Day 7 to Day 14}}$$

Data Analysis

Pre-treatment body weight data was first tested for equal variance using Levene's Test (α = 0.05) (Weber, 1989). A 2-sample t-test was run to determine whether there were differences in weight between sexes and between treatments. Mean measured body weights, calculated body weight change and weekly feed consumption per bird per day were similarly analyzed at the end of the study. Data were statistically analyzed using Mini Tab 17 (Minitab, Inc., College Station, PA).

The nominal oral limit dose tested in this limit test and corresponding mortality data were used to empirically estimate whether the median lethal dose (LD_{50}) and the No Observed Effect Level (NOEL) were greater or less than 2000 mg a.i./kg body weight.

Results

Behavioral Observations and Mortality

No mortality, abnormal behavior or symptoms of toxicity were observed during this study. The mortality data for this study are presented in Table 215.

Post-mortem Examinations

No abnormal or unusual findings were noted among the birds examined at study termination, which included all birds in the study.

Body Weight

Pre-treatment body weights (Day -1) were of equal variance with no statistically significant mean differences between male and female birds nor between treatment and control birds. Therefore, subsequent analyses were conducted with sexes combined.

There were no significant mean differences in measured body weights recorded on Day 7 or Day 14 post-dosing.

Calculated body weight change from Day -1 to Day 7 (Week 1) and from Day 8 to Day 14 (Week 2) were not significantly different between the control group and treatment group.

Body weight data collected during this study are summarized in **Table 216**.

Feed Consumption

There were no statistically significant differences in feed consumption per bird per day between the control and treatment groups during Week 1 or Week 2 of the study. Feed consumption data collected during this study are summarized in Table 217.

CONCLUSION

The test satisfied the following acceptance criteria:

- ✓ Birds were randomly assigned to treatment and control pens.
- ✓ ≤ 10 % of the control birds died or became moribund during the test: *actual = 0 %*
- ✓ A minimum of 10 birds are used for the control group and the limit dose group: *actual = 10 per group*
- ✓ Birds were orally administered the test substance, via intubation or capsules: *actual = intubation*
- ✓ A minimum of 10 birds were dosed at the limit dose of 2,000 mg a.i./kg body weight: *actual = 10 birds.*

No mortality, abnormal behavior or symptoms of intoxication were observed during the study after dosing 10 birds at the limit dose of 2000 mg recombinant IPD072Aa Protein /kg body weight. Food consumption, mean measured body weight and calculated change in body weight did not differ between treatment and control groups at any time point during the study. The results of this study empirically demonstrate the northern bobwhite median lethal dose (LD50) and the no effect dose level are greater than 2000 mg a.i./kg body weight.

Table 214. Doses administered to Northern Bobwhite (*Colinus virginianus*) during the acute oral toxicity test with recombinant IPD072Aa protein.

Group	Cage #	Sex	Bird Weight (kg)	Target Dose (mg/kg)	Test Substance Purity	Target Test Substance (mg) / Bird	Target Test Substance (g)/bird
Control	6	M	0.1865	2000	1	373	0.3730
Control	8	F	0.1761	2000	1	352.2	0.3522
Control	10	F	0.1938	2000	1	387.6	0.3876
Control	12	M	0.1967	2000	1	393.4	0.3934
Control	14	M	0.1886	2000	1	377.2	0.3772
Control	16	F	0.1855	2000	1	371	0.371
Control	18	M	0.1675	2000	1	335	0.335
Control	23	F	0.1653	2000	1	330.6	0.3306
Control	25	M	0.1821	2000	1	364.2	0.3642
Control	26	F	0.1855	2000	1	371	0.371
Treatment	1	M	0.1742	2000	0.8	435.5	0.4355
Treatment	3	F	0.1928	2000	0.8	482	0.482
Treatment	4	F	0.1822	2000	0.8	455.5	0.4555
Treatment	7	F	0.1906	2000	0.8	476.5	0.4765
Treatment	9	M	0.1876	2000	0.8	469	0.469
Treatment	11	F	0.1672	2000	0.8	418	0.418
Treatment	13	F	0.1723	2000	0.8	430.75	0.43075
Treatment	15	M	0.1787	2000	0.8	446.75	0.44675
Treatment	17	M	0.193	2000	0.8	482.5	0.4825
Treatment	19	M	0.1969	2000	0.8	492.25	0.49225

Table 215. Summary of survival of northern bobwhite (*Colinus virginianus*) during the acute oral toxicity limit test with recombinant IPD072Aa protein

Dose (mg/kg body weight)	Cumulative Dead by Study Day		Total # Dead	Total % Mortality
	0 - 7	8 - 14		
0	0	0	0	0
2000	0	0	0	0

Table 216. Body weight data collected during the northern bobwhite (*Colinus virginianus*) acute oral toxicity limit test with recombinant IPD072Aa protein

Dose (mg ai/kg body weight)	Cage No.	Sex	Body Weight Day -1 (g)	Body Weight Day 7 (g)	Body Weight Day 14 (g)	Day -1 to Day 7 weight change (g)	Day 7 to Day 14 weight change (g)	Total Change in Body Weight (g)
0	8	Female	176.1	187.7	190.6	11.6	2.9	14.5
0	10	Female	193.8	201.3	202.2	7.5	0.9	8.4
0	16	Female	185.5	191.4	193.0	5.9	1.6	7.5
0	23	Female	165.3	174.5	181.5	9.2	7	16.2
0	26	Female	185.5	194.3	195.1	8.8	0.8	9.6
0	6	Male	186.5	193.5	192.6	7.0	-0.9	6.1
0	12	Male	196.7	205.2	208.7	8.5	3.5	12.0
0	14	Male	188.6	193.1	195.9	4.5	2.8	7.3
0	18	Male	167.5	175.1	176.7	7.6	1.6	9.2
0	25	Male	182.1	191.4	194.2	12.2	2.8	12.1
2000	3	Female	192.8	203.4	206.3	10.6	2.9	13.5
2000	4	Female	182.2	189.8	193.1	7.6	3.3	10.9
2000	7	Female	190.6	202.7	207.5	12.1	4.8	16.9
2000	11	Female	167.2	176.7	179.9	9.5	3.2	12.7
2000	13	Female	172.3	180.8	185.5	8.5	4.7	13.2
2000	1	Male	174.2	181.8	178.2	7.6	-3.6	4.0
2000	9	Male	187.6	196.4	199.4	8.8	3.0	11.8
2000	15	Male	178.7	184.9	185	6.2	0.1	6.3
2000	17	Male	193.0	199.5	202.1	6.5	2.6	9.1
2000	19	Male	196.9	207.0	209.1	10.1	2.1	12.2

Table 217. Summary of feed consumption data collected during the northern bobwhite (*Colinus virginianus*) acute oral toxicity limit test with recombinant IPD072Aa protein

Dose (mg ai/kg feed)	Cage No.	Sex	Week 1 consumed/bird/day (g)	Week 2 consumed/bird/day (g)
0	8	F	20.1	18.67
0	10	F	20.67	18.77
0	16	F	18.63	17.74
0	23	F	26.66	22.79
0	26	F	21.69	19.79
0	6	M	19.93	18.66
0	12	M	23.34	21.27
0	14	M	21.00	18.67
0	18	M	20.19	18.50
0	25	M	22.30	19.84
2000	3	F	23.31	22.50
2000	4	F	20.76	19.13
2000	7	F	22.56	20.23
2000	11	F	22.79	19.94
2000	13	F	20.63	17.93
2000	1	M	19.61	17.27
2000	9	M	21.09	17.93
2000	15	M	21.03	20.54
2000	17	M	22.36	20.36
2000	19	M	20.73	19.46

G8. IPD072Aa Protein: Acute Oral Toxicity Study in Mice

MATERIALS AND METHODS

Test Substance

IPD072Aa protein was supplied by Pioneer Hi-Bred International, Inc. (Pioneer) and assigned number 31739. The bulk test substance was maintained at <-50ºC for long-term storage or up to one month opened at <-10ºC. Purity and composition of the test substance was characterized by Pioneer.

Control Substance

BSA (Sigma Aldrich, St. Louis, Missouri, U.S.A., catalog number A3059, CAS 9048-46-8) was obtained by the testing facility and assigned number 30181. BSA was stored in accordance with specifications provided by the manufacturer. Purity and composition were characterized by the manufacturer.

Test System

Male and female (nulliparous and non-pregnant) Crl:CD1(ICR) mice were received from Charles River Laboratories International, Inc., Raleigh, North Carolina, U.S.A.

Mice have historically been used in safety evaluation studies. The Crl:CD1(ICR) mouse was selected based on consistently acceptable health status and on extensive experience with the strain at the contract research organization (CRO).

Animal Husbandry

Housing

During the in-life phase, animals were housed individually (males) or in pairs (females) in solid-bottom caging with bedding and appropriate species-specific enrichment. Each cage rack contained only animals of one sex.

Environmental Conditions

Animal rooms were maintained at a temperature of 20-26ºC (68-79ºF) and a relative humidity of 30-70%. Animal rooms were artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle. Excursions outside of these ranges were of insufficient magnitude and/or duration to have adversely affected the validity of the study. Animal rooms had a minimum of 10 complete fresh air changes per hour.

Feed and Water

Tap water was available *ad libitum*. PMI® Nutrition International, LLC Certified Rodent LabDiet® 5002 was available *ad libitum* except during the fasting period.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and not to exceed stated maximum concentrations of key contaminants, including specified heavy metals, aflatoxin, chlorinated hydrocarbons, and organophosphates. The presence of these contaminants below the maximum concentration stated by the manufacturer would not be expected to impact the integrity of the study.

Identification

Each mouse was assigned an identification number, which was written on each animal's tail with a water-insoluble marker.

Acclimation

Animals were weighed and observed for general health during the 6-day quarantine period.

Animal Health and Environmental Monitoring Program

As specified in the CRO animal health and environmental monitoring program, the following procedures are performed periodically to ensure that contaminant levels are below those that would be expected to impact the scientific integrity of the study:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.

The animal health and environmental monitoring program is administered by the attending laboratory animal veterinarian. Evaluation of these data did not indicate any conditions that affected the validity of the study.

Assignment to Study

The animals were selected for use based on adequate body weight gain and freedom from clinical signs of disease or injury. At study start, the animals were approximately 7 weeks of age (young adult). The weight variation of selected animals did not exceed ± 20% of the mean weight for each sex.

Dose Preparation and Administration

IPD072Aa protein and BSA protein were each reconstituted in deionized water on a weight/volume basis. The IPD072Aa dose formulation was adjusted for IPD072Aa concentration of 820 µg/mg. The BSA dose formulation was not adjusted for purity. Dose formulations were not analyzed quantitatively for homogeneity or accuracy of concentration. The dosing mixtures were gently stirred throughout the dosing procedure.

The vehicle control, BSA control and IPD072Aa test substance formulations were administered orally by gavage on test day 1 at a volume of 20 mL/kg body weight. The target dose level for the BSA control group and IPD072Aa test substance-treated group was 2000 mg/kg. Individual dose volumes were calculated based on the individual fasted body weights determined on the day of dosing.

The mice were fasted approximately 4 hours prior to administration on test day 1. Food was returned to the animals approximately one hour after dosing.

Body Weights

Individual weights were recorded at the start of fasting (test day 1), shortly before the test substance was administered (test day 1), and on test days 2, 3, 5, 8, and 15.

Clinical Observations and Mortality Checks

Careful Clinical Observations

Each animal was removed from its cage, individually handled and examined for abnormal behavior and/or appearance:

- Prior to fasting on test day 1
- Prior to dosing
- Approximately 30 minutes after dosing
- Approximately 2 hours after dosing
- Once daily thereafter

The observation records over the course of the study included a description of any observed clinical signs, the time of onset and duration of the clinical signs, and the time of death.

Daily Animal Health Observations

In addition to the above-specified clinical observations, each animal was observed for mortality/moribundity twice daily, once in the morning and once in the afternoon.

Disposition of Animals

All animals were euthanized by exsanguination while under isoflurane anesthesia and given a complete gross pathological examination. The examination included:

- general appearance and health
- all orifices
- cranial, thoracic and abdominal cavities and contents

The complete GI tract from each animal was also excised and preserved. Because no gross lesions were observed at necropsy, the GI tract will be discarded.

RESULTS AND DISCUSSION

In-life Toxicology

Body Weights, Clinical Observations, and Survival

There were no instances of mortality, clinical abnormalities, or overall (test day 1-15) losses in body weight observed in any animals during the study (Table 218; Table 219;Table 220;Table 221).

Anatomic Pathology Evaluation

Gross Observations

No gross lesions were present in the mice at necropsy.

CONCLUSIONS

Under the conditions of this study, intragastric exposure of IPD072Aa protein to male and female mice at 2000 mg/kg did not result in mortality or other evidence of acute oral toxicity, based on evaluation of body weight, clinical signs, and gross pathology. Therefore, the LD_{50} of IPD072Aa protein was determined to be greater than 2000 mg/kg.

Table 218. Mean Body Weights of Male Mice

Bodyweight (g)

Sex: Male Day(s) Relative to Start Date		Vehicle Control	2000 mg/kg BSA Cont	2000 mg/kg IPD072Aa
1	Mean	27.8	28.0	29.1
	SD	2.2	1.0	2.3
	N	6	6	6
2	Mean	28.7	29.1	30.3
	SD	2.2	1.0	2.5
	N	6	6	6
3	Mean	28.7	29.2	30.5
	SD	2.1	1.0	2.5
	N	6	6	6
5	Mean	29.6	29.9	31.4
	SD	2.2	0.9	2.7
	N	6	6	6
8	Mean	30.3	30.2	32.0
	SD	2.6	0.8	2.6
	N	6	6	6
15	Mean	32.4	31.6	33.9
	SD	3.0	1.1	2.9
	N	6	6	6

Table 219. Mean Body Weights of Female Mice

Bodyweight (g)

Sex: Female Day(s) Relative to Start Date		Vehicle Control	2000 mg/kg BSA Cont	2000 mg/kg IPD072Aa
1	Mean	24.0	24.1	23.9
	SD	1.1	0.7	1.0
	N	6	6	6
2	Mean	24.8	25.2	25.4
	SD	1.6	1.0	1.1
	N	6	6	6
3	Mean	24.7	25.4	25.7
	SD	1.5	0.8	1.0
	N	6	6	6
5	Mean	25.2	25.8	25.3
	SD	1.3	0.9	1.3
	N	6	6	6
8	Mean	25.5	25.6	26.3
	SD	1.3	0.5	1.1
	N	6	6	6
15	Mean	27.2	26.5	26.5
	SD	2.0	0.8	1.5
	N	6	6	6

Table 220. Mean Body Weight Gains of Male Mice

Body Weight Gain (g)

Sex: Male Day(s) Relative to Start Date		Vehicle Control	2000 mg/kg BSA Cont	2000 mg/kg IPD072Aa
1 → 8	Mean	2.5	2.2	2.9
	SD	0.7	0.8	0.9
	N	6	6	6
8 → 15	Mean	2.1	1.4	1.9
	SD	0.7	0.3	0.6
	N	6	6	6
1 → 15	Mean	4.6	3.6	4.8
	SD	1.1	0.9	1.1
	N	6	6	6

Table 221. Mean Body Weight Gains of Female Mice

Body Weight Gain (g)

Sex: Female Day(s) Relative to Start Date		Vehicle Control	2000 mg/kg BSA Cont	2000 mg/kg IPD072Aa
1 → 8	Mean	1.5	1.5	2.4
	SD	0.9	0.7	0.1
	N	6	6	6
8 → 15	Mean	1.7	0.9	0.2
	SD	1.1	0.8	0.8
	N	6	6	6
1 → 15	Mean	3.2	2.4	2.6
	SD	1.7	1.3	0.7
	N	6	6	6

Table 222. Summary of Clinical Observations and Mortality in Male Mice

Day numbers relative to Start Date

Sex: Male

	Vehicle Control	2000 mg/kg BSA Cont	2000 mg/kg IPD072Aa
Scheduled sacrifice			
Number of Observations	6	6	6
Number of Animals	6	6	6
Days from - to	15 15	15 15	15 15

Table 223. Summary of Clinical Observations and Mortality in Female Mice

Day numbers relative to Start Date

Sex: Female

	Vehicle Control	2000 mg/kg BSA Cont	2000 mg/kg IPD072Aa
Scheduled sacrifice			
Number of Observations	6	6	6
Number of Animals	6	6	6
Days from - to	15 15	15 15	15 15

Appendix H. Estimated Environmental Concentration (EEC) and Margin of Exposure (MOE) Calculations

Problem formulation was used to develop hypotheses of potential harm to NTOs and the environment, based on knowledge of the receiving environment, the biology of the crop, and the characteristics of the introduced insecticidal traits. Problem formulation was used to guide the exposure and hazard assessments, so that the ERA is informative and predictive of risk (Carstens et al., 2010; Raybould, 2006; Romeis et al., 2013; Wolt and Peterson, 2010).

Worst-case estimated environmental concentrations (EECs) of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize were determined using worst-case assumptions (i.e., maximum concentrations of DvSSJ1 dsRNA and the IPD072Aa protein in relevant DP23211 maize tissues) to determine potential exposure for NTOs, including pollinators and pollen feeders, soil-dwelling organisms, aquatic organisms, predators and parasitoids, granivorous mammals and insectivorous birds. Refined EECs were calculated under more realistic assumptions of environmental conditions to understand environmentally relevant concentrations.

Early-tier laboratory studies were conducted using representative surrogate species from several functional guilds at concentrations that exceeded the EEC. For each surrogate species, the median lethal concentration (LC_{50}), lethal dose (LD_{50}), no-observed-effect-concentration (NOEC), no-observed-effect-dose (NOED), or no-observed-effect-dietary-dose (NOEDD) was determined for relevant endpoints (e.g., mortality, adult weight) for both the DvSSJ1 dsRNA and the IPD072Aa protein and compared to the worst-case EEC or refined EEC to determine the margin of exposure (MOE). An MOE that exceeds 10X the EEC is considered conservative and is indicative of minimal risk under realistic environmental conditions (Rose, 2007). All MOE calculations were conducted based on the dry weight concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize tissues. The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017). Insect bioassays may be reported based on wet weight or dry weight concentrations, and all details related to experimental design are provided in the main body of the petition.

H1. Pollinators and Pollen Feeders

One key consideration in the exposure assessment of pollen feeders and pollinators is the concentration of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen. In a comprehensive field expression study, the mean concentration of the DvSSJ1 dsRNA was 9.87 x 10^{-4} ng/mg pollen (ranging from 5.61 x 10^{-4} – 2.02 x 10^{-3} ng/mg pollen dry weight. The mean

IPD072Aa protein concentration was 0.65 ng/mg pollen (ranging from 0.14 – 1.3 ng/mg pollen dry weight.

Honey bees

Worst-case EECs were calculated for honey bee exposure to the DvSSJ1 dsRNA and the IPD072Aa protein in maize pollen.

Honey bee are exposed to 100% of the maximum concentration of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen (**Table 224**).

The amount of pollen consumed is assumed to be 2 mg for honey bee larvae (Babendreier et al., 2004) and 4.3 mg for honey bee adults (Crailsheim et al., 1992).

Refined EEC were not calculated for honey bee, due to the low exposure under the worst-case EEC assumptions.

Table 224. Worst-case EEC for Honey Bee Larvae and Adults Exposed to the DvSSJ1 dsRNA and the IPD072Aa Protein in DP23211 Maize Pollen

	Pollen Concentration[1]		Worst-case EEC	
	Mean (ng/mg)	Maximum (ng/mg)	Larvae[2] (ng/larvae)	Adults[3] (ng/bee)
DvSSJ1 dsRNA	9.87×10^{-4}	2.02×10^{-3}	4.04×10^{-3}	8.69×10^{-3}
IPD072Aa Protein	0.65	1.3	2.6	5.59

[1] For the purposes of calculating an EEC, the concentration of the DvSSJ1 dsRNA and the IPD072Aa protein is calculated using the maximum pollen tissue concentration (dry weight). The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017).
[2] A honey bee larvae is assumed to ingests 2 mg of pollen during development (Babendreier et al., 2004).
[3] A honey bee adult is assumed to ingests 3.4-4.3 mg of pollen (Crailsheim et al., 1992). To be conservative, 4.3 mg of pollen was used for calculating EECs.

MOE calculations for honey bee larvae exposed to the DvSSJ1 dsRNA in DP23211 maize pollen

The worst-case EEC for honey bee larvae exposed to the DvSSJ1 dsRNA in DP23211 maize pollen is 4.04×10^{-3} ng/larvae.

Honey bee larvae were used in a 22 day bioassay (following OECD Guidance Document No. 239) with a targeted concentration of 0.0040 µg DvSSJ1 dsRNA/larva (section E2. Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure to DvSSJ1 210bp dsRNA). No effects on larval

survival, pupal survival, adult emergence, or adult weight at emergence were observed. The NOED for honey bee larvae is 0.0040 µg DvSSJ1 dsRNA/larval cell (equivalent to 4.0 ng DvSSJ1 dsRNA/larvae cell).

The MOE, based on a NOED of 4.0 ng DvSSJ1 dsRNA/larvae, is 990X the worst-case EEC for honey bee larvae exposed to the DvSSJ1 dsRNA in DP23211 maize pollen.

MOE calculations for honey bee adults exposed to the DvSSJ1 dsRNA in DP23211 maize pollen

The worst-case EEC for honey bee adults exposed to the DvSSJ1 dsRNA in DP23211 maize pollen is 8.69×10^{-3} ng/bee.

Honey bee adults (≤ 2-day old emerged) were exposed (following OECD 245) to a mean daily dose of 0.026 µg DvSSJ1 dsRNA, per bee per day, for 14 days (section E3. 14-Day Oral Toxicity Test with the Adult Honey Bee (*Apis mellifera*) Exposed to DvSSj1 210bp dsRNA). No effects on adult body weight or survival were observed. The NOEDD for honey bee adults is 0.026 µg DvSSJ1 dsRNA/bee/day (equivalent to 26 ng DvSSJ1 dsRNA/bee/day).

The MOE, based on a NOEDD of 26 ng DvSSJ1 dsRNA/bee is 2,993X the worst-case EEC for honey bee adults exposed to DvSSJ1 RNA in DP23211 maize.

MOE calculations for honey bee larvae exposed to the IPD072Aa protein in DP23211 maize pollen

The worst-case EEC for honey bee larvae exposed to the IPD072Aa protein in DP23211 maize pollen is 2.6 ng/larvae.

Honey bee larvae were used in a 22 day bioassay (following OECD Guidance Document No. 239) with targeted concentrations of 0.10 and 0.20 µg IPD072Aa protein/larva (section G2. IPD072Aa Protein: Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure). No effects on larval survival, pupal survival, adult emergence, or adult weight at emergence were observed. The NOED for honey bee larvae is 0.20 µg IPD072Aa protein/larval cell (equivalent to 200 ng IPD072Aa protein/larvae cell).

The MOE, based on a NOED of 200 ng IPD072Aa protein/larvae, is 77X the worst-case EEC for honey bee larvae exposed to the IPD072Aa protein in DP23211 maize pollen.

MOE calculations for honey bee adults exposed to the IPD072Aa protein in DP23211 maize pollen

The worst-case EEC for honey bee adults exposed to the IPD072Aa protein in DP23211 maize pollen is 5.59 ng/bee.

Honey bee adults (≤ 2-day old emerged) were exposed (following OECD 245) to a mean daily dose of 1.3 µg IPD072Aa protein, per bee per day, for 10 days (section G3. IPD072Aa Protein: 10-Day Oral Toxicity Test with the Adult Honey Bee (Apis mellifera)). No effects on adult body weight or survival were observed. The NOEDD for honey bee adults is 1.3 µg IPD072Aa protein/bee/day (equivalent to 1,300 ng IPD072Aa protein/bee/day).

The MOE, based on a NOEDD of 1,300 ng IPD072Aa protein/bee, is 233X the worst-case EEC for honey bee adults exposed to the IPD072Aa protein in DP23211 maize pollen.

H2. Non-Target Lepidoptera

Worst-case EECs were calculated for non-target Lepidoptera exposure to the DvSSJ1 dsRNA and the IPD072Aa protein in maize pollen.

Non-target Lepidoptera are exposed to 100% of the maximum concentration of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize pollen (Table 225).

Refined EEC were not calculated for non-target Lepidoptera, due to the low exposure under the worst-case EEC assumptions; however, other factors are expected to further limit the magnitude and duration of exposure (host plant density, pollen deposition rates, feeding behavior, spatial and temporal overlap, etc).

Table 225. Worst-case EEC for Non-Target Lepidoptera Exposed to the DvSSJ1 dsRNA and the IPD072Aa Protein in DP23211 Maize Pollen

	Pollen Concentration[1]		Worst-case EEC[2] (ng/mg DW)
	Mean (ng/mg)	Maximum (ng/mg)	
DvSSJ1 dsRNA	9.87×10^{-4}	2.02×10^{-3}	2.02×10^{-3}
IPD072Aa Protein	0.65	1.3	1.3

[1] The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017).
[2] For the purposes of calculating an EEC, the concentration of the DvSSJ1 dsRNA and the IPD072Aa protein is calculated using the maximum pollen tissue concentration (dry weight).

MOE calculations for non-target Lepidoptera exposed to DvSSJ1 dsRNA by incidental feeding to the DP23211 maize pollen

The worst-case EEC for non-target Lepidoptera exposed to DvSSJ1 dsRNA in DP23211 maize pollen is 2.02×10^{-3} ng/mg.

Tier I hazard studies on non-target Lepidoptera for the DvSSJ1 dsRNA were not conducted based on negligible potential for exposure.

The LC_{50} of DvSSJ1 dsRNA for WCR is 0.036 ng/mg (D1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing DvSSJ1_210 double stranded RNA), which is 18X higher than the worst-case EEC for non-target Lepidoptera.

MOE calculations for non-target Lepidoptera exposed to IPD072Aa protein by incidental feeding to the DP23211 maize pollen

The worst-case EEC for non-target Lepidoptera exposed to the IPD072Aa protein in DP23211 maize pollen is 1.3 ng/mg.

Tier I hazard studies on non-target Lepidoptera for the IPD072Aa protein were not conducted based on negligible potential for exposure.

The LC_{50} of the IPD072Aa protein for WCR is 26 ng/mg (F1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing IPD072Aa Protein), which is 20X higher than the worst-case EEC for non-target Lepidoptera.

H3. Soil-dwelling Organisms

The DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize may enter the soil through root exudates, root sloughing, pollen deposition, and post-harvest plant tissue decomposition. Soil-dwelling organisms may be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via ingestion of DP23211 senescent maize tissues (detritivores).

Worst-case EECs were calculated for soil-dwelling organisms exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via consumption of plant material (detritivores).

The worst-case EEC for soil-dwelling organisms that consume senescent plant material can be calculated based on the maximum concentration of DvSSJ1 dsRNA and the IPD072Aa protein in senescent (R6) whole plant tissue (Table 226).

Refined EECs were calculated for soil-dwelling organisms exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via consumption of plant material (detritivores).

A refined EEC for soil-dwelling decomposers and detritivores can be calculated based on the mean concentration of DvSSJ1 dsRNA and the IPD072Aa protein in senescent (R6) whole plant tissue (Table 226).

Table 226. Worst-case and Refined EEC for Soil-Dwelling Organisms that Consume Plant Material (Detritivores)

	Tissue Concentration (R6 Whole plant)[1]		Soil Detritivore EECs	
	Mean (ng/mg)	Maximum (ng/mg)	Worst-case (ng/mg)	Refined (ng/mg)
DvSSJ1 dsRNA	1.08×10^{-2}	2.99×10^{-2}	2.99×10^{-2}	1.08×10^{-2}
IPD072Aa Protein	11	24	24	11

[1]The concentration of the DvSSJ1 dsRNA and the IPD072Aa protein is reported based on dry weight. The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017).

MOE calculations for non-target soil dwelling organisms exposed to DvSSJ1 dsRNA from DP23211 maize.

The worst-case EEC for soil dwelling organisms exposed via consumption senescent maize tissue is 2.99×10^{-2} ng/mg. The refined EEC for soil dwelling organisms exposed via consumption senescent maize tissue is 1.08×10^{-2} ng/mg.

Springtail adults were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 28 days (section E1. Evaluation of the Survival and Reproduction of Springtail Fed an Artificial Diet Containing DvSSJ1 210bp dsRNA). No adverse effect on springtail reproduction or survival were observed.

The MOEs, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 33X the worst-case EEC and 93 X the refined EEC.

MOE calculations for non-target soil dwelling organisms exposed to the IPD072Aa protein from DP23211 maize.

The worst-case EEC for soil dwelling organisms exposed via consumption senescent maize tissue is 24 ng/mg. The refined EEC for soil dwelling organisms exposed via consumption senescent maize tissue is 11 ng/mg.

Springtail adults were exposed to diet containing a target concentration of 500 ng IPD072Aa protein/mg diet for 28 days (section G1. Evaluation of the Survival and Reproduction of Springtail Fed an Artificial Diet Containing IPD072Aa Protein). No biologically relevant adverse effect on springtail reproduction and no effects on survival were observed.

The MOE, based on a NOEC of 500 ng IPD072Aa protein/mg diet, is 21X the worst-case EEC and 45 X the refined EEC.

H4. Aquatic Organisms

Potential exposure of non-target aquatic organisms to plant incorporated protectants in GM crops has been considered previously with movement of senescent tissue identified as the most likely route of exposure for aquatic organisms (Carstens et al., 2010). Although aquatic habitats may be located near agricultural areas, exposure of aquatic organisms to biotech crops is limited temporally and spatially (Bachman et al., 2016) and aquatic exposure to *Bt* corn is extremely small (US-EPA, 2010). The specificity and environmental fate of the DvSSJ1 dsRNA and the IPD072Aa protein, as well as the worst-case assumptions about potential input of maize tissue in aquatic environments, can be used to help inform the risk assessment for aquatic organisms (Carstens et al., 2012).

The worst-case EEC for aquatic organisms to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize tissues was estimated using the EPA standard agricultural field-farm pond model (also called the US EPA standard pond model (Jones et al., 2004)). The EPA standard agricultural field-farm pond model provides estimates for predicting pesticide runoff concentrations and uses the assumptions that runoff from a 10-hectare (ha) field is deposited in a 1-ha pond (2 meters deep; equivalent to 20,000,000 L of water).

Worst-case EECs were calculated for aquatic organisms using the EPA standard agricultural field-farm pond model

A pond (1-ha, 2 meters deep, containing 20,000,000 L of water) receives 100% of the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize from a 10-ha field.

One maize plant weighs 0.3 kg dry weight; maize density is 75,000 plants/ha; equivalent to 22,500 kg plant tissue/ha (Carstens et al., 2012).

DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize tissue are freely-soluble and instantaneously bioavailable. No degradation occurs in the field or the pond.

The highest mean whole plant tissue concentration (highest mean value from any growth stage) of DvSSJ1 dsRNA and the IPD072Aa protein are used to be most conservative (Table 227).

Table 227. Worst-case EECs for Aquatic Organisms Determined Using the EPA Standard Agricultural Field-Farm Pond Model

	Tissue Concentration (ng/mg)[1]	Worst-case EEC (mg/l)[2]
DvSSJ1 dsRNA	2.19×10^{-2}	2.46×10^{-4}
IPD072Aa Protein	11	0.124

[1] The concentration of the DvSSJ1 dsRNA and the IPD072Aa protein is reported based on dry weight. The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017).

[2] For the purposes of calculating an EEC, the highest mean whole plant tissue concentration (highest mean value from any growth stage across the growing season) was used to determine EECs; for DvSSJ1 dsRNA, the R1 whole plant tissue had the highest mean value; for the IPD072Aa protein, R6 whole plant tissue had the highest mean value.

MOE calculations for aquatic organisms exposed to DvSSJ1 dsRNA from DP23211 maize

The worst-case EEC for aquatic organisms exposed to DvSSJ1 dsRNA in DP23211 maize is 2.46×10^{-4} mg/l (ppm).

Tier I hazard studies on aquatic species for the DvSSJ1 dsRNA were not conducted based on negligible potential for exposure.

The LC_{50} of DvSSJ1 dsRNA for WCR is 0.036 ng/mg, which is 146X higher than the worst-case EEC for aquatic organisms (section D1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing DvSSJ1_210 double stranded RNA.).

MOE calculations for aquatic organisms exposed to IPD072Aa protein from DP23211 maize

The worst-case EEC for aquatic organisms exposed to the IPD072Aa protein in DP23211 maize is 0.124 mg/l (ppm).

Tier I hazard studies on aquatic species for the IPD072Aa protein were not conducted based on negligible potential for exposure.

The LC_{50} of the IPD072Aa protein for WCR is 26 ng/mg which is 210X higher than the worst-case EEC for aquatic organisms (section F1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing IPD072Aa Protein).

H5. Predators and Parasitoids

A predator or parasitoid may be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via consumption of prey that has previously consumed tissue from a DP23211 maize plant.

Worst-case EECs were calculated for predators and parasitoids exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via prey.

Predators are exposed to the maximum concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (maximum from any above-ground plant tissue and from any growth stage; Table A5).

100% of the dsRNA or protein in the GM plant transfers to the prey and then subsequently is transferred to the predator (no degradation or loss).

Refined EECs were calculated for predators and parasitoids exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via prey.

Predators are exposed to the mean concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (highest mean value from any above-ground plant tissue and from any growth stage; Table 228).

100% of the dsRNA or protein in the GM plant transfers to the prey and then subsequently is transferred to the predator (no degradation or loss).

Table 228. Worst-case and Refined EEC for Predators and Parasitoids

	Tissue Concentration[1]		EEC[2]	
	Mean (ng/mg DW)	Maximum (ng/mg DW)	Worst-case (ng/mg DW)	Refined (ng/mg DW)
DvSSJ1 dsRNA	0.0646	0.113	0.113	0.0646
IPD072Aa Protein	16	39	39	16

[1] The concentration of the DvSSJ1 dsRNA and the IPD072Aa protein is reported based on dry weight. The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017).

[2] For the purposes of calculating a worst-case EEC, the maximum concentration in above-ground tissue at any growth stage was used; for DvSSJ1 dsRNA leaf (R4) tissue had the highest maximum value; for the IPD072Aa protein, the leaf (V9) tissue had the highest maximum value. To calculate the refined EEC, the mean concentration (highest mean concentration in above-ground tissue at any growth stage) was used; for DvSSJ1 dsRNA, leaf (R4) tissue had the highest mean value; for the IPD072Aa protein, the leaf (R1) tissue had the highest mean value.

H5.1. Green Lacewing

Green lacewing larvae (*Chrysoperla* species) are generalist predators that may inhabit maize field and field margins. The most likely route of exposure of green lacewing larvae to DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize plants is via secondary transfer via prey (predatory route). Green lacewing adults are pollinators and pollen feeders. However, because the larval stage is considered the most sensitive, and because the EECs based on a predatory route of exposure are higher than those based on a pollen feeder route of exposure, an EEC based on larval exposure to the DvSSJ1 dsRNA and the IPD072Aa protein via secondary transfer is considered most protective of the population.

MOE calculations for Green Lacewing exposed to DvSSJ1 dsRNA via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.113 ng/mg. The refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.0646 ng/mg

Green Lacewing larvae were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 21 days (section E5. Evaluation of the Survival and Development of Green Lacewing Fed an Artificial Diet Containing DvSSJ1 210bp dsRNA). No adverse effects on survival or pupation of green lacewing were observed.

The MOE, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 9X the worst-case EEC and 15X the refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey.

MOE calculations for Green Lacewing exposed to the IPD072Aa protein via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey is 39 ng/mg. The refined EEC for predators and parasitoids exposed to IPD072Aa protein in DP23211 maize via prey is 16 ng/mg.

Green Lacewing larvae were exposed to diet containing a target concentration of 500 ng IPD072Aa protein/mg diet for 21 days (section G5. Evaluation of the Survival and Development of *Green Lacewing* Fed). No adverse effects on survival or pupation of green lacewing were observed.

The MOE, based on a NOEC of 500 ng IPD072Aa protein/mg diet, is 13X the worst-case EEC and 31X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey.

H5.2. Ladybird Beetles

Ladybird beetles (Family Coccinellidae) can be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize via direct feeding on pollen (pollinivorous species), via indirect ingestion of pollen while feeding on other food sources, or via secondary transfer (predatory species). For the purposes of calculating an EEC, the predatory route of exposure results in higher EECs than the pollen-feeder route of exposure. Therefore, the predatory route of exposure was used to determine the worst-case EEC for ladybird beetles exposed to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize.

MOE calculations for CMAC exposed to DvSSJ1 dsRNA via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.113 ng/mg. The refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.0646 ng/mg.

CMAC neonates were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 28 days (section F9. Evaluation of the Survival and Development of Pink Spotted Lady Beetle Fed Artificial Diets Containing IPD072Aa Protein). No adverse effects on survival, weight, or number of days to adult emergence were observed.

The MOE, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 9X the worst-case EEC and 15X the refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey

MOE calculations for CMAC exposed to the IPD072Aa protein via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey is 39 ng/mg. The refined EEC for predators and parasitoids exposed to IPD072Aa protein in DP23211 maize via prey is 16 ng/mg.

CMAC neonates were exposed to diet containing a target concentration of 100, 500, and 1000 ng IPD072Aa protein/mg diet for 28 days (section F9. Evaluation of the Survival and Development of Pink Spotted Lady Beetle Fed Artificial Diets Containing IPD072Aa Protein). No adverse effects on survival, weight, or number of days to adult emergence were observed at 100 ng IPD072Aa protein/mg diet.

The MOE, based on a NOEC of 100 ng IPD072Aa protein/mg diet, is 3X the worst-case EEC and 6X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein/mg in DP23211 maize via prey.

MOE calculations for CNV exposed to DvSSJ1 dsRNA via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.113 ng/mg. The refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.0646 ng/mg

CNV neonates were exposed to diet containing a target concentration of 1 ng DvSSJ1 dsRNA/mg diet for 28 days (section E6. Evaluation of the Survival, Weight, and Development of Convergent Lady Beetle Fed an Artificial Diet Containing DvSSJ1 210bp dsRNA). No adverse effects on survival, weight, or adult emergence were observed.

The MOE, based on a NOEC of 1 ng DvSSJ1 dsRNA/mg diet, is 9X the worst-case EEC and 15X the refined for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey.

MOE calculations for CNV exposed to the IPD072Aa protein via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey is 39 ng/mg. The refined EEC for predators and parasitoids exposed to IPD072Aa protein in DP23211 maize via prey is 16 ng/mg.

CNV neonates were exposed to diet containing a target concentration of 100 ng, 500 ng, and 1000 ng IPD072Aa protein/mg diet for 28 days (section G6. Evaluation of the Survival and Development of Convergent Lady Beetle Fed Artificial Diets Containing IPD072Aa Protein). Exposure to 100 ng and 500 ng IPD072Aa protein/mg diet had no adverse effects on survival.

The MOE, based on a survival NOEC of 500 ng IPD072Aa protein/mg diet, is 13X the worst-case EEC and 31X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey.

H5.3. Parasitic Hymenoptera

Parasitic Hymenoptera can be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize via secondary transfer (predatory species) if their larval hosts are feeding on DP23211 tissue. For the purposes of calculating an EEC, the predatory route of exposure was used to derive the EECs.

MOE calculations for Parasitic Hymenoptera exposed to DvSSJ1 dsRNA via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.113 ng/mg. The refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.0646 ng/mg

Parasitic Hymenoptera were exposed to diet containing a target concentration of 1 µg DvSSJ1 dsRNA/ml diet for 14 days (section E4. Evaluation of the Survival of Parasitic Hymenoptera Fed Artificial Diets Containing DvSSJ1 210 bp dsRNA). No adverse effects on survival were observed.

The MOE, based on a NOEC of 1 µg DvSSJ1 dsRNA/ml diet, is 9X the worst-case EEC and 15X the refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey.

MOE calculations for Parasitic Hymenoptera exposed to IPD072Aa protein via a predator route of exposure

The worst-case EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey is 39 ng/mg. The refined EEC for predators and parasitoids exposed to IPD072Aa protein in DP23211 maize via prey is 16 ng/mg.

Parasitic Hymenoptera were exposed to diet containing a target concentration of 100, 500, and 1000 µg IPD072Aa protein/ml diet for 7 days (section G4. Evaluation of the Survival of Parasitic Hymenoptera Fed Artificial Diets Containing IPD072Aa Protein). No adverse effects on survival were observed.

The MOE, based on a NOEC of 1000 µg IPD072Aa protein/ml diet, is 26X the worst-case EEC and 63 X the refined EEC for predators and parasitoids exposed to the IPD072Aa protein in DP23211 maize via prey.

H6. Insectivorous Birds

Some wild birds are insectivorous and could be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via prey (tri-tropic transfer). The factors that may limit the potential exposure of wild birds to the DvSSJ1 dsRNA and the IPD072Aa protein via prey are discussed above for predators and parasitoids.

Worst-case EECs were calculated for insectivorous birds exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via prey.

Predators are exposed to the maximum concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (maximum from any above-ground plant tissue and from any growth stage; Table 229).

100% of the dsRNA or protein in the GM plant transfers to the prey and then subsequently is transferred to the predator (no degradation or loss).

Refined EECs were calculated for insectivorous birds exposed to the DvSSJ1 dsRNA and the IPD072Aa protein via prey.

Predators are exposed to the mean concentration of the DvSSJ1 dsRNA and the IPD072Aa protein expressed in tissue (highest mean value from any above-ground plant tissue and from any growth stage; Table 229).

100% of the dsRNA or protein in the GM plant transfers to the prey and then subsequently is transferred to the predator (no degradation or loss).

Table 229. Worst-case and Refined EEC for Insectivorous Birds via a Predator Route of Exposure

	Tissue concentration[1]		EEC[2]	
	Mean (ng/mg DW)	Maximum (ng/mg DW)	Worst-case (ng/mg DW)	Refined (ng/mg DW)
DvSSJ1 dsRNA	0.0646	0.113	0.113	0.0646
IPD072Aa Protein	16	39	39	16

[1] The concentration of the DvSSJ1 dsRNA and the IPD072Aa protein is reported based on dry weight. The dry weight concentrations are considered high estimates, since in reality NTOs would be exposed to levels comparable to fresh weight levels (US-EPA, 2017).

[2] For the purposes of calculating a worst-case EEC, the maximum concentration in above-ground tissue at any growth stage was used; for DvSSJ1 dsRNA leaf (R4) tissue had the highest maximum value; for the IPD072Aa protein, the leaf (V9) tissue had the highest maximum value. To calculate the refined EEC, the mean concentration (highest mean concentration in above-ground tissue at any growth stage) was used; for DvSSJ1 dsRNA, leaf (R4) tissue had the highest mean value; For the IPD072Aa protein, the leaf (R1) tissue had the highest mean value.

MOE calculations for wild birds that are exposed to DvSSJ1 dsRNA via an insectivorous route of exposure

The worst-case EEC for insectivorous birds exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.113 ng/mg. The refined EEC for predators and parasitoids exposed to DvSSJ1 dsRNA in DP23211 maize via prey is 0.0646 ng/mg.

Colinus virginianus were exposed to a nominal limit dose of 105 mg DvSSJ1 dsRNA/kg body weight for 14 days (section E7. Northern Bobwhite (*Colinus virginianus*) Acute Oral Toxicity Limit Test with RNA oligonucleotide DvSSJ1_210 dsRNA)(Stanfield, 2019)(Stanfield, 2019)(Stanfield, 2019)(Stanfield, 2019)(Stanfield, 2019)(Stanfield, 2019)(Stanfield, 2019)(Stanfield, 2019)(Stanfield, 2019) . No mortality, abnormal behavior or signs of toxicity were observed.

The MOE, based on a NOEL and LD_{50} >105 mg DvSSJ1 dsRNA/kg body weight, is 929X the worst-case EEC and 1,625X the refined EEC for wild birds that are exposed via an insectivorous route of exposure (predator).

MOE calculations for wild birds that are exposed to IPD072Aa protein via an insectivorous route of exposure

The worst-case EEC for insectivorous birds exposed to the IPD072Aa protein in DP23211 maize via prey is 39 ng/mg. The refined EEC for predators and parasitoids exposed to IPD072Aa protein in DP23211 maize via prey is 16 ng/mg.

C. virginianus were exposed to a nominal limit dose of 2000 mg IPD072Aa protein/kg body weight for 14 days (section G7. Northern Bobwhite (*Colinus virginianus*) Acute Oral Toxicity Limit Test with Recombinant IPD072Aa Protein). No mortality, abnormal behavior or signs of toxicity were observed.

The MOE, based on a NOEC and LD_{50} of >2000 mg IPD072Aa protein/kg body weight, is 51X the worst-case EEC and 125X the refined EEC for wild birds that are exposed via an insectivorous route of exposure (predator).

H7. Granivorous Mammals

Granivorous wildlife (e.g., rodents) may be exposed to the DvSSJ1 dsRNA and the IPD072Aa protein by feeding on DP23211 maize grain.

Worst-case EECs were calculated for wild mammals that are exposed via grain feeding

Maximum concentration of DvSSJ1 dsRNA and the IPD072Aa protein in DP23211 maize grain (R6).

73% of wild mammal diet is maize grain (Raybould et al., 2007).

A daily dietary dose (DDD) is calculated, which accounts for food intake, body weight, and DvSSJ1 dsRNA and the IPD072Aa protein concentration in grain, where DDD = FIR/BW * C (Crocker et al., 2002; Raybould et al., 2007).

The worst-case FIR/BW ratio for seed-eating rodents is 0.33 for the harvest mouse (*Micromys minutus*) (Raybould et al., 2007).

Table A7. Worst-case and Refined EEC for Wild Mammals that are Exposed via Grain Feeding

	Maximum Tissue Concentration[1]	Worst-case EEC[2]
	Grain (R6) (ng/mg DW)	Mammals (mg/kg body weight)
DvSSJ1 dsRNA	1.09×10^{-2}	2.6×10^{-3}
IPD072Aa Protein	4.8	1.156

[1] For the purposes of calculating an EEC, the maximum grain concentration (R6) is used.
[2] The refined EEC for mammals assumes the worst-case FIR/BW ratio (0.33 kg tissue/kg body weight); 73% of diet is maize grain. Example calculation: DDD = (FIR/BW * grain concentration) * % maize in diet = (0.33 mg tissue/kg body weight * 4.8 ng IPD072Aa protein/mg grain) * 73%) = 1.156 mg IPD072Aa protein/kg body weight.

MOE calculations for wild mammals that are exposed to IPD072Aa protein via a grain feeding route of exposure

The worst-case EEC for wild mammals exposed to the IPD072Aa protein in DP23211 maize is 1.156 mg/kg body weight.

M. musculus were orally exposed at a dose of 2000 mg IPD072Aa protein/kg body weight for 14 days (section G8. IPD072Aa Protein: Acute Oral Toxicity Study in Mice) (Table 4). No mortality or other evidence of acute oral toxicity was observed, based on evaluation of body weight, clinical signs, and gross pathology. The LD_{50} for *M. musculus* was determined to be >2000 mg IPD072Aa/kg body weight.

The MOE, based on an LD_{50} >2000 mg IPD072Aa/kg body weight, is 1730X the worst-case EEC for wild mammals exposed via the grain feeding route of exposure.

Appendix I. Using Diabrotica virgifera virgifera to Evaluate the Combined Potency ofIPD072Aa Protein and DvSSJ1_210 double-stranded RNA

Materials

Test Substances

IPD072Aa Protein

This test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under -80 °C freezer unit storage condition.

DvSSJ1_210 dsRNA

This test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under-80 °C freezer unit storage condition.

Carrier

The carrier consisted primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Dosing Solutions

The bioassay control dosing solution used to prepare Treatment 1 consisted of RNase-free water.

Test dosing solutions used to prepare Treatments 2-8 consisted of IPD072Aa protein and/or DvSSJ1_210 dsRNA test substances diluted in RNase-free water to achieve the concentrations in the test diets.

Test System

The test system was *Diabrotica virgifera virgifera* (western corn rootworm; Coleoptera: Chrysomelidae). The test system was chosen because *WCR* is an insect sensitive to IPD072Aa protein and DvSSJ1_210 dsRNA. *WCR* eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel.

Experimental Design

Dose response curves with WCR (14-day duration) for each test substance were generated outside of this study and used to estimate the LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50} of each single

active. The independent model of combined action (Bliss, 1939) was used to estimate the potency of a mixture of these two test substances. The independent action model is appropriate to assess the combined potency in this study given the different modes of action between these two test substances (protein and RNA interference (RNAi); (Borgert *et al.*, 2004; US-EPA, 2009).

Assuming independent action, concentrations were selected to elicit a range of responses of WCR to a mixture of the two test substances. Three independent bioassays were conducted exposing WCR larvae via oral ingestion to the treatments listed in Table 230.

Within each bioassay, treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3 replicates from each treatment. Each treatment in each bioassay was fed to a target of 30 *WCR* individuals. The bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed every 3 to 4 days. After each 14-day bioassay, mortality was assessed, and surviving organisms were individually weighed.

Quantitative assays (enzyme-linked immunosorbent assay (ELISA) or QuantiGene) were used to verify the concentration of IPD072Aa protein or DvSSJ1_210 dsRNA, respectively, in working stock solutions, dosing solutions, and/or spiking solutions, as appropriate. The homogeneity of IPD072Aa protein or DvSSJ1_210 dsRNA in the highest dose treatment was also verified. Stability of the test substances under bioassay conditions was previously verified over four days for IPD072Aa protein (Appendix X, Section A) and DvSSJ1_210 dsRNA and was not re-confirmed in this study.

The bioassay acceptability criterion indicated a bioassay may be terminated and repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment 1) group. Treatments 2 and 3 are each expected to have an observed mortality of approximately 50% and serve as additional bioassay controls to detect large differences in the expected performance of *WCR*.

Bias in the *WCR* bioassays was controlled through the randomization of treatments within blocks and the use of control diet. Control of bias during sample collection and analysis was achieved through the use of replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. In addition, the use of working stock solutions and stabilized curve preparation minimized day to day bias.

Diet Generation, Sample Collection, and Diet Characterization

Diet Generation

On each day of diet preparation, dosing solutions were individually prepared as described in the Analytical Phase section below. Dosing solutions for Treatments 2-8 were prepared from IPD072Aa protein and/or DvSSJ1_210 dsRNA working stock solutions; Treatment 1 dosing solutions consisted of RNase-free water. Each dosing solution was mixed with carrier in a 2.51:1 ratio (*i.e.*, 2.51 ml of dosing solution to 1 g of carrier), generating Treatments 1-8.

Sample Collection and Diet Characterization

During the process of diet distribution, samples of Treatments 1 and 8 were collected for characterization as described in the Analytical Phase section below.

WCR Bioassay

Bioassays were conducted to determine the response of WCR to IPD072Aa protein and/or DvSSJ1_210 dsRNA exposure via oral ingestion. A separate batch of WCR eggs for each bioassay was incubated in an environmental chamber until the eggs hatched. Neonates were used in each bioassay within 24 hours of hatching.

On Day 0 of each respective bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diet was dispensed into wells of the bioassay plates. One WCR neonate was placed in each well containing diet. Each plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for a total of 14 days. Every 3 to 4 days, new bioassay plates were prepared with fresh diet as described for Day 0, living WCR larvae were transferred to the new plates, missing or dead organisms were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, each bioassay was complete, mortality was assessed, and surviving organisms were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well or lost in transfer, or wells containing more than one organism, were excluded from statistical analysis.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4.

To correct for differences between bioassays, data were adjusted prior to model fitting. For each bioassay, observed mortality rates were adjusted by the natural mortality of the bioassay control diet treatment using a modification of Abbott's adjustment as shown below:

$$Deaths_{adj} = n * (P_T - P_C)$$

$$n_{adj} = n * (1 - P_C)$$

Where $Deaths_{adj}$ refers to the adjusted number of dead insects in a given treatment, n refers to the number of insects observed, P_T refers to the observed mortality rate of the treatment, P_C refers to the mortality rate of the bioassay control diet treatment, and n_{adj} refers to the adjusted number of insects observed.

A generalized linear model assuming a binomial distribution and a logit link function was used to fit the adjusted mortality data as responding to treatment. Mortality percentages for each treatment were estimated with 95% confidence intervals.

Results and Discussion

Two of the three *WCR* bioassays met the acceptability criterion as dead and missing organisms in the bioassay control diet (Treatment 1) group did not exceed 30%. The third bioassay did not meet the acceptability criterion as combined dead and missing organisms exceeded 30%. However, eight organisms were missing and mortality alone was 27.3% (Table 231); therefore, the bioassay was not repeated and the results are included in this study. The corrected mortality of Treatments 2 and 3, targeting the LC_{50} of IPD072Aa protein and DvSSJ1_210 dsRNA, respectively, across the three bioassays was approximately 50% (47.3% and 58.2%, respectively; Table 231) and confidence intervals for these estimates included 50%. These results indicate the response of WCR used in this study is consistent with the response of WCR in studies of similar design used to generate the independent dose-response curves for IPD072Aa protein (section D1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing DvSSJ1_210 double stranded RNA) and DvSSJ1_210 dsRNA (section F1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing IPD072Aa Protein).

For Treatments 2 through 5, the 95% confidence intervals for estimated mortality encompassed the mortality predicted by the independent model of combined action. For Treatments 6 and 7, the 95% confidence intervals did not contain the expected level of mortality derived from the independent action model and 100% mortality was observed for Treatment 8.

A five-fold increase in potency was considered as a threshold beyond which additional work would be necessary to further characterize synergism (US-EPA, 2009). As illustrated in Table 232, the upper 95% confidence limits for observed mortality in Treatments 4-7 are less than the expected mortality for hypothetical mixtures demonstrating 2X synergism. Thus, it can be concluded that the potency of various mixtures of IPD072Aa protein and DvSSJ1_210 dsRNA is demonstrating less than 2X synergism and well below the 5X threshold considered in this study as necessary for further characterization of the synergistic response (see Appendix C). In Treatment 8, observed mortality reached 100% which made comparisons to a hypothetically synergistic mixture impossible. However, WCR demonstrated a dose-dependent response with each of the mixture combinations at lower concentrations and it is reasonable to conclude a lack of a biologically significant response for this treatment. Further, Belden and Brain (2018) have suggested the independent model of combined action may under-estimate toxicity of mixtures, perhaps due to generalized physiological effects within organisms exposed to combinations of stressors.

ELISA or QuantiGene analysis verified the concentration of IPD072Aa protein or DvSSJ1_210 dsRNA, respectively, in the working stock solutions used to prepare the dosing solutions for Treatments 2-8 for each bioassay. Further, the concentration of IPD072Aa protein was verified in the dosing solutions used to prepare Treatment 8 for each bioassay. DvSSJ1_210 dsRNA concentration was verified in the spiking solution used to prepare Treatment 8 for each bioassay. The homogeneity of IPD072Aa protein or DvSSJ1_210 dsRNA in Treatment 8 was also verified. Characterization results are presented in Table 233 and Table 234.

A summary of WCR larval weight data is provided in Table 236.

Conclusion

Based on the observations noted in this study, there is no evidence of a biologically relevant synergistic effect with IPD072Aa protein and DvSSJ1_210 dsRNA. The observed potency of the mixtures in this study followed a dose-dependent and predictable response and were below the levels suggested to indicate continued characterization of the potency of the mixture (US-EPA, 2009). Therefore, safety studies conducted with each of the single test substances are informative for environmental risk assessments.

Table 230. WCR IPD072Aa and DvSSJ1 dsRNA Treatment Descriptions

Treatment ID	Treatment Description	Target Concentration (ng IPD072Aa/mg)	Target Concentration (ng DvSSJ1/mg)
1	Bioassay Control Diet	0	0
2	LC_{50} of IPD072Aa	14.0	0
3	LC_{50} of DvSSJ1	0	0.036
4	LC_{10} of each test substance	6.30	0.0018
5	LC_{20} of each test substance	8.46	0.0054
6	LC_{30} of each test substance	10.3	0.011
7	LC_{40} of each test substance	12.1	0.021
8	LC_{50} of each test substance	14.0	0.036

Note: Targeted IPD072Aa protein and/or DvSSJ1_210 dsRNA concentrations in Treatments 2-8 are based on diet wet weight.

Table 231. Summary Analysis of WCR IPD072Aa Protein and DvSSJ1 dsRNA Mortality Results

Treatment	Treatment Description	Bioassay	Total Number of Observations [a]	Total Number of Dead Organisms	Mortality (%)	Mortality with Abbott's correction (%)	Estimated Mortality (%) with Abbott's Correction (95% Confidence Interval)	Predicted Mortality (%)
1	Bioassay Control Diet [b]	1	28	5	17.9	0	--	0
		2	29	0	0	0		
		3	22	6	27.3	0		
2	LC_{50} of IPD072Aa	1	28	12	42.9	30.4	47.3 (34.3 - 60.7)	50
		2	27	14	51.9	51.9		
		3	20	15	75.0	65.6		
3	LC_{50} of DvSSJ1	1	27	16	59.3	50.4	58.2 (45.0 - 70.3)	50
		2	29	23	79.3	79.3		
		3	25	13	52.0	34.0		
4	LC_{10} of each test substance	1	28	8	28.6	13.0	20.5 (11.9 - 33.0)	19
		2	28	7	25.0	25.0		
		3	25	11	44.0	23.0		
5	LC_{20} of each test substance	1	28	12	42.9	30.4	38.0 (26.2 - 51.4)	36
		2	28	15	53.6	53.6		
		3	23	10	43.5	22.3		
6	LC_{30} of each test substance	1	27	17	63.0	54.9	71.3 (58.6 - 81.4)	51
		2	30	25	83.3	83.3		
		3	29	23	79.3	71.6		
7	LC_{40} of each test substance	1	26	21	80.8	76.6	80.7 (68.1 - 89.1)	64
		2	30	26	86.7	86.7		
		3	22	18	81.8	75.0		
8	LC_{50} of each test substance	1	25	25	100	100	100[c]	75
		2	30	30	100	100		
		3	24	24	100	100		

[a] Organisms counted as missing during the bioassay or lost in transfer, or wells containing more than one organism, were not included in the total number of observations for a given treatment.

[b] Bioassay acceptability criteria is dead and missing organism count ≤ 30%.

[c] Due to 100% mortality, mortality was not corrected and no confidence interval was generated.

Table 232. Comparison of Observed Mortality with that Expected at a 2-fold Greater Concentration of the Mixture

Treatment ID	Treatment Description	IPD072Aa Protein Concentration	DvSSJ1_210 dsRNA Concentration	2X IPD072Aa Protein Concentration	2X DvSSJ1_210 dsRNA Concentration	Expected Mortality at 2X IPD072Aa Protein Concentration	Expected Mortality at 2X DvSSJ1_210 dsRNA Concentration	Expected Mortality with Mixture at 2X Concentration	Upper 95% Confidence Limit of Observed Mortality for Mixture
4	LC_{10}	6.3	0.0018	12.6	0.0035	42.6	15.6	51.5	33
5	LC_{20}	8.5	0.0054	16.9	0.0107	62.5	29.3	73.5	51.4
6	LC_{30}	10.3	0.011	20.6	0.0225	74.1	41.5	84.9	81.4
7	LC_{40}	12.1	0.021	24.2	0.0412	81.7	52.5	91.3	89.1
8	LC_{50}	14.0	0.036	28.1	0.0719	87.0	62.4	95.1	NA

Note: Not applicable (NA).

Table 233. Verification of IPD072Aa Protein Concentration in Working Stock and Test Dosing Solutions and Homogeneity in Test Diet

Bioassay	Time Point	% of Target	Result
Working Stock Solution Concentration Verification[a]			
1	Day of Preparation	94	Verified
	After Final Use	88	Verified
2	Day of Preparation	92	Verified
	After Final Use	89	Verified
3	Day of Preparation	91	Verified
	After Final Use	104	Verified
Treatment 8 Dosing Solution Concentration Verification[a]			
1	Day 0	95	Verified
2	Day 11	79	Verified
3	Day 0	86	Verified
Treatment 8 Homogeneity Verification[b]			
1	Day 0	NA	Verified
2	Day 11	NA	Verified
3	Day 0	NA	Verified

Note: Not applicable (NA).

[a] Working stock and dosing solution concentrations within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified for a given bioassay and treatment if the value of each sample was within 70-130% of the mean value of all samples analyzed for that bioassay and treatment.

Table 234. Verification of DvSSJ1_210 dsRNA Concentration in Working Stock and Test Spiking Solutions and Homogeneity in Test Diet

Bioassay	Time Point	% of Target	Result
Working Stock Solution Concentration Verification[a]			
1-3	Day of Preparation	113	Verified
	After Final Use	109	Verified
Treatment 8 Spiking Solution Concentration Verification[a]			
1	Day 4	121	Verified
2	Day 8	109	Verified
3	Day 11	120	Verified
Treatment 8 Homogeneity Verification[b]			
1	Day 4	NA	Verified
2	Day 8	NA	Verified
3	Day 11	NA	Verified

Note: Not applicable (NA).

[a] Working stock and spiking solution concentrations within 70-130% of the expected value were considered verified.

[b] Homogeneity was considered verified for a given bioassay and treatment if the value of each sample was within 70-130% of the mean value of all samples analyzed for that bioassay and treatment.

I.1. WCR DvSSJ1 dsRNA and IPD072Aa Protein Analytical Phase

The following dosing solutions were prepared for the *Diabrotica virgifera virgifera (WCR*) bioassays:

Bioassay control dosing solution used to prepare Treatment 1 consisting of RNase-free water

Test dosing solutions used to prepare Treatments 2-8 consisting of IPD072Aa protein and/or DvSSJ1_210 dsRNA test substances diluted in RNase-free water to achieve the concentrations in the test diets

Quantitative assays (enzyme-linked immunosorbent assay (ELISA) or QuantiGene) were used to verify the concentration of IPD072Aa protein or DvSSJ1_210 dsRNA, respectively, in working stock solutions, dosing solutions, and/or spiking solutions, as appropriate. The homogeneity of IPD072Aa protein or DvSSJ1_210 dsRNA in Treatment 8 was also verified. As each test substance was combined in the same dosing solution prior to incorporation into the diet, either analyte could serve as an indication of diet homogeneity. Homogeneity assessment of one test diet on two days of diet preparation for each bioassay is considered representative of all days of diet preparation for all test diets.

Preparation of Solutions

An appropriate volume of each test substance was thawed under chilled conditions or at ambient temperature, as applicable, then separately diluted in RNase-free water to create IPD072Aa protein and DvSSJ1_210 dsRNA working stock solutions (targeted concentrations 19.6 ng/µl and 1510 ng/µl, respectively). Individual IPD072Aa protein working stock solutions were prepared prior to each bioassay; the DvSSJ1_210 dsRNA working stock solution was prepared in bulk and used across bioassays. When not in use, the working stock solutions were stored in a 2-8 °C refrigerator unit or -80°C freezer unit (for IPD072Aa protein and DvSSJ1_210 dsRNA, respectively).

On each day of diet preparation for a bioassay, the IPD072Aa protein working stock solution was serially diluted in RNase-free water to create IPD072Aa protein dosing solutions for Treatments 4-7 and used undiluted to prepare IPD072Aa protein dosing solutions for Treatments 2 and 8. The DvSSJ1_210 dsRNA working stock solution was removed from the freezer, allowed to thaw, and then serially diluted in RNase-free water to create DvSSJ1_210 dsRNA spiking solutions for Treatments 3-8. The DvSSJ1_210 dsRNA spiking solutions were added to the IPD072Aa protein dosing solutions to prepare combined IPD072Aa protein/DvSSJ1_210 dsRNA dosing solutions for Treatments 4-8. DvSSJ1_210 dsRNA spiking solution was added directly to RNase-free water to create the dosing solution for Treatment 3. The IPD072Aa protein concentrations in the test dosing solutions ranged from 8.81-19.6 ng/µl

and the DvSSJ1_210 dsRNA concentrations in the test dosing solutions ranged from 0.0025-0.050 ng/µl. The bioassay control dosing solution consisted of RNase-free water. Dosing solutions were prepared and maintained chilled.

Characterization of IPD072Aa Protein Working Stock Solutions

The concentration of IPD072Aa protein in each working stock solution was verified for the day of preparation and following final use using a quantitative ELISA method that had been internally validated to demonstrate method suitability. Samples were interpolated using a standard curve with the IPD072Aa protein test substance as the analytical standard.

IPD072Aa Protein ELISA Method

Prior to analysis, samples were diluted as applicable in chilled 25% StabilZyme Select in phosphate-buffered saline containing polysorbate 20 (PBST). Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with an IPD072Aa-specific antibody. Following incubation, unbound substances were washed from the plate. A different IPD072Aa-specific antibody, conjugated to the enzyme horseradish peroxidase (HRP), was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound IPD072Aa-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader.

Determination of Protein Concentration

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the OD values obtained for each set of sample wells to a protein concentration value.

A standard curve was included on each ELISA plate. The equation for the standard curve was derived by the software, which used a quadratic fit to relate the OD values obtained for each set of standard wells to the respective standard concentration (ng/ml).

The quadratic regression equation was applied as follows: $y = Cx^2 + Bx + A$

where x = known standard concentration and y = respective absorbance value (OD)

Interpolation of the sample concentration (ng/ml) was performed by solving for x in the above equation using the values for A, B, and C that were determined for the standard curve.

$$\text{Sample Concentration (ng/ml)} = \frac{-B + \sqrt{B^2 - 4C(A - \text{sample OD})}}{2C}$$

For example, given curve parameters of A = 0.0476, B = 0.4556, C= -0.01910, and a sample OD = 1.438

Sample Concentration = $\frac{-0.4556+\sqrt{0.4556^2-4(-0.01910)(0.0476-1.438)}}{2(-0.01910)}$ = 3.6 ng/ml

The sample concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the interpolated concentration by N.

Adjusted Concentration = Interpolated Sample Concentration x Dilution Factor

For example, given an interpolated concentration of 3.6 ng/ml and a dilution factor of 1:20

Adjusted Concentration = 3.6 ng/ml x 20 = 72 ng/ml

Verification of IPD072Aa Protein Concentration in the Working Stock Solution

The concentration of IPD072Aa protein in the working stock solution was considered verified if the results fell within 70-130% of the expected value.

Characterization of DvSSJ1_210 dsRNA Working Stock Solutions

The concentration of DvSSJ1_210 dsRNA in the working stock solution was verified for the day of preparation and following final use using QuantiGene analysis. Samples were diluted in QuantiGene Homogenizing Solution (QHS) 1:10,000, and stored frozen (-80 °C freezer unit) until analysis, if applicable.

QuantiGene Analysis

The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies. The DvSSJ1 QuantiGene Plex Assay method utilized beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. A signal amplification tree was built on the LE probes by incubation with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances.

Once the signal amplification tree was complete, each well was incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the Treatment 8 working stock solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, homogeneity), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Working Stock Solution

The concentration of DvSSJ1_210 dsRNA in the working stock solution was considered verified if the results fell within 70-130% of the expected value.

Characterization of Test Dosing Solutions

On one day of diet preparation for each bioassay, the concentration of IPD072Aa protein in the dosing solution used to create Treatment 8 was verified using an ELISA method, as described in Section B. On a different day of diet preparation for each bioassay, the concentration of DvSSJ1_210 dsRNA in the spiking solution used to create Treatment 8 was verified using QuantiGene analysis, as described in Section C, with the exception that samples were diluted 1:100 in QHS. IPD072Aa protein dosing solution samples were analyzed on the day of collection; DvSSJ1_210 dsRNA spiking solution samples were stored frozen (-80 °C freezer unit) until analysis, as applicable.

Characterization of Diets

Diet Sample Collection

During the process of diet distribution for the *WCR* bioassays, homogeneity samples were collected as shown in Table 235. Five samples of Treatment 1 were also collected twice per bioassay for verification of test substance absence but those samples were not analyzed. Collected samples were stored frozen (-80 °C freezer unit) until analysis, if applicable (Table 235).

Table 235. WCR IPD072Aa and DvSSJ1 dsRNA Diet Samples

Analysis	Bioassay	Diet Preparation Day	Analyte	Number of Samples
Homogeneity of Treatment 8	1	0	IPD072Aa Protein	15 per assessment[a]
		4	DvSSJ1_210 dsRNA	
	2	8	DvSSJ1_210 dsRNA	
		11	IPD072Aa Protein	
	3	0	IPD072Aa Protein	
		11	DvSSJ1_210 dsRNA	

[a] Five samples were collected at the beginning of the diet distribution process, five in the middle, and five at the end for each homogeneity assessment.

Preparation of Sample Homogenates

Collected diet samples for homogeneity (three each from the beginning, middle, and end of diet distribution for each assessment) were prepared for extraction and analysis as follows:

For IPD072Aa protein, sub-samples were weighed to approximately 40 mg on wet ice prior to extraction in 600 µl of 25% StabilZyme Select in chilled PBST. Samples were analyzed on the day of extraction.

For DvSSJ1_210 dsRNA, QHS (3000 µl) was added to each tube containing diet (~300 mg) and samples were vortexed continuously for at least four minutes, and centrifuged. The supernatants were centrifuged again and the final supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable.

Homogeneity Assessment

Homogeneity analysis was conducted using the previously described ELISA or QuantiGene methods with the following calculations.

The mean concentration $\overline{x}$ of test substance, expressed in ng/ml or MFI, was determined across all samples analyzed for a given test diet.

The acceptable range was calculated, using the following equation:

Acceptable range = $\overline{x} \times (1 \pm 0.3)$

Since no samples were observed falling outside of the acceptable range, the 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated using binomial distribution:

$$\mathrm{UL} = 1 - (1 - 0.95)^{1/n}$$

where *n* was the number of samples in each verification.

The 95% upper confidence limit for the probability of a sample falling outside of the acceptable range was calculated and determined to be 0.283. Therefore, testing 9 samples was determined to be sufficient to conclude homogeneity.

Summary of Diabrotica virgifera virgifera Larval Weight Data

Table 236. Summary of *Diabrotica virgifera virgifera* Larval Weight Data

Treatment ID	Treatment Description	Number of Surviving Organisms[a]	Weight (mg)	
			Mean ± Standard Deviation	Range (mg)
1	Bioassay Control Diet	68	1.50 ± 0.723	0.2 - 3.0
2	LC_{50} of IPD072Aa	34	0.226 ± 0.116	0.1 - 0.5
3	LC_{50} of DvSSJ1	29	0.641 ± 0.500	0.1 - 1.9
4	LC_{10} of each test substance	55	0.411 ± 0.391	0.1 - 2.9
5	LC_{20} of each test substance	42	0.293 ± 0.128	0.1 - 0.5
6	LC_{30} of each test substance	21	0.281 ± 0.150	0.1 - 0.6
7	LC_{40} of each test substance	13	0.262 ± 0.145	0.1 - 0.6
8	LC_{50} of each test substance	0	NA	NA

Not applicable (NA); there were no surviving *Diabrotica virgifera virgifera* in Treatment 8.
[a] Across the three bioassays.

Further Description of The Model Deviation Ratio Approach

A five-fold increase in potency was considered as a threshold beyond which additional work would be necessary to further characterize synergism (US-EPA, 2009). One way to mathematically define this threshold is illustrated by a Model Deviation Ratio (MDR) equal to 5 (Belden and Lydy, 2006). That is, the effective concentration of the mixture predicted by the model (independent action in this case), divided by the effective concentration observed from toxicity testing.

To use this threshold based on the MDR, we have assumed that a synergistic effect is equivalent to multiplying the effective concentration of each single active by some factor below the threshold of 5. A factor of 2 was used in this instance to give a hypothetically synergistic mixture demonstrating 2X synergism. This assumption is necessary, as the independent action model for dissimilar modes of action does not assume parallel dose response curves. Multiplying each of the single active concentrations by 2 and then generating expected responses for the single actives and for the mixture allowed for comparison of the observed mortality of the mixtures used in this study against a hypothetical mixture demonstrating 2X synergism (Table 232).

Estimated dose response curves for each of the single actives were generated by first estimating the LC_{50} and slope for each individual dose response curve characterized in section D1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing DvSSJ1_210 double stranded RNA, and F1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing IPD072Aa Protein (Figure 97; Figure 98; Figure 99; Figure 100). A random-effects meta-analysis approach was then used to derive an estimate of the overall mean LC_{50} as described in section I.2. IPD072Aa 14 day LC50 with WCR (Table 237; Table 238). The same approach was used to estimate the overall mean slope associated with each of the meta-analysis generated dose-response curves. These two parameter estimates were used to generate the expected dose-response curves for each single active using the following formula:

$$\pi_j = 100 * \frac{e^{\beta \log_{10} \frac{x_j}{\theta}}}{1 + e^{\beta \log_{10} \frac{x_j}{\theta}}}$$

where π_j denotes the expected percent mortality at concentration x_j, β denotes the estimated slope, and θ denotes the estimated LC_{50}.

The independent model of combined action was then used to generate an expected mortality value for the various mixtures of the two test substances used in this study. These expected mortality values associated with each single active and the mixtures are reported in Table 232.

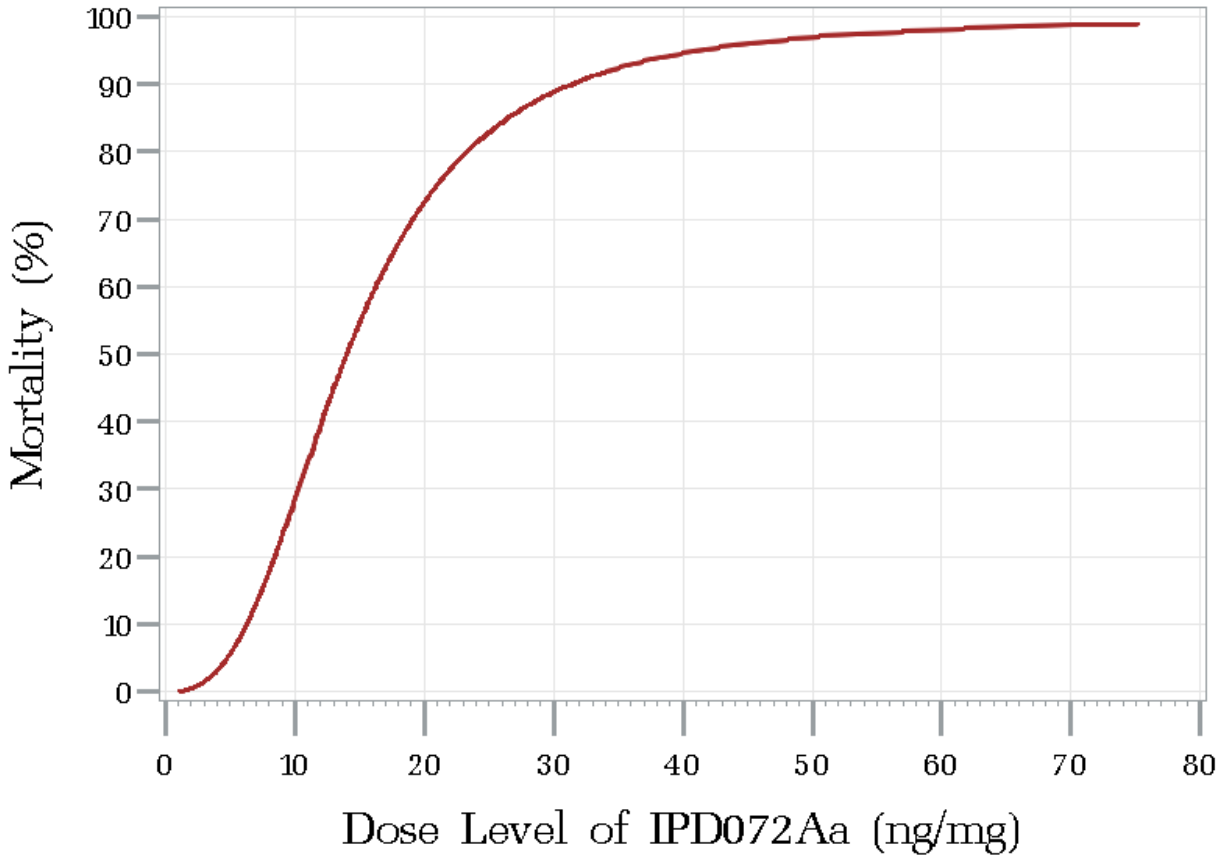


Figure 97. Meta-analysis Dose-response Curve for Bioassays with IPD072Aa Protein Reported in section F1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing IPD072Aa Protein

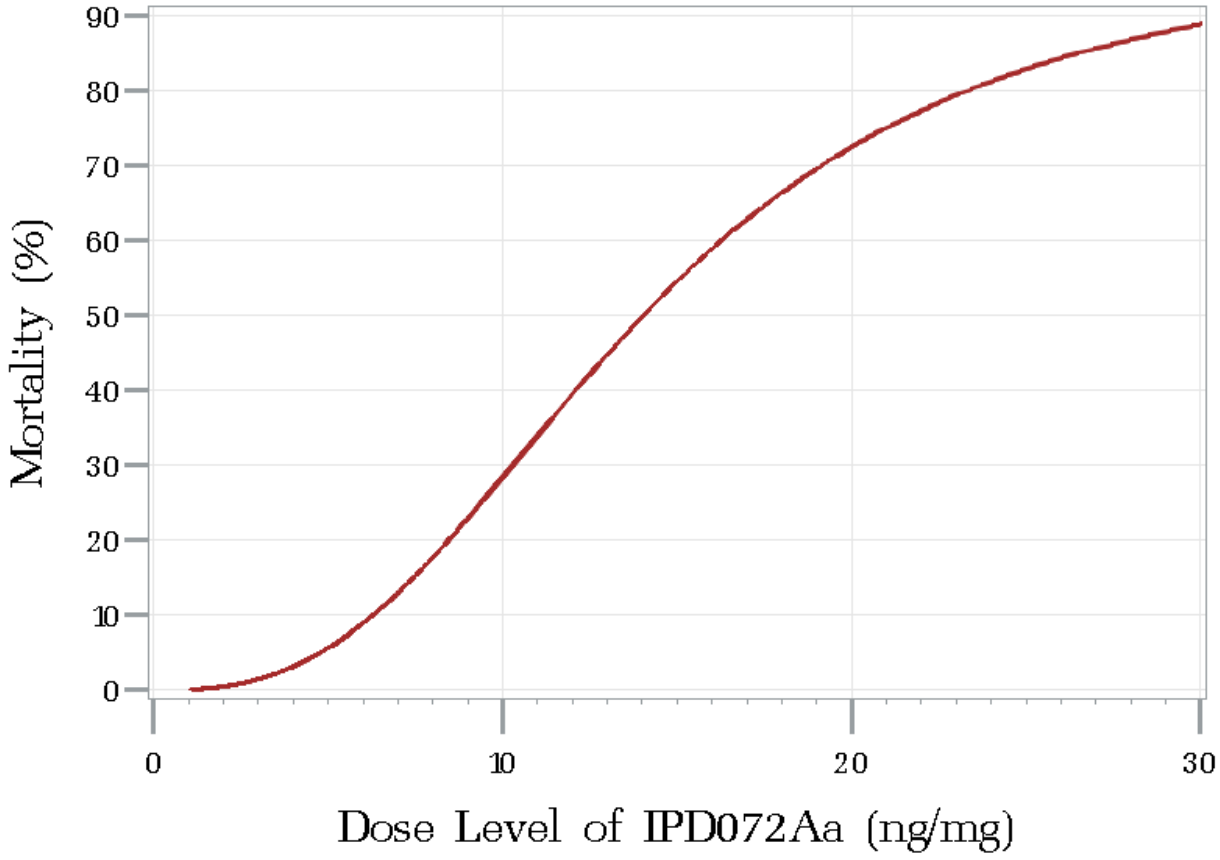


Figure 98. Meta-analysis Dose-response Curve for Bioassays with IPD072Aa Protein Showing Greater Resolution of the Estimated Mortality Between 0 and 30 ng/mg.

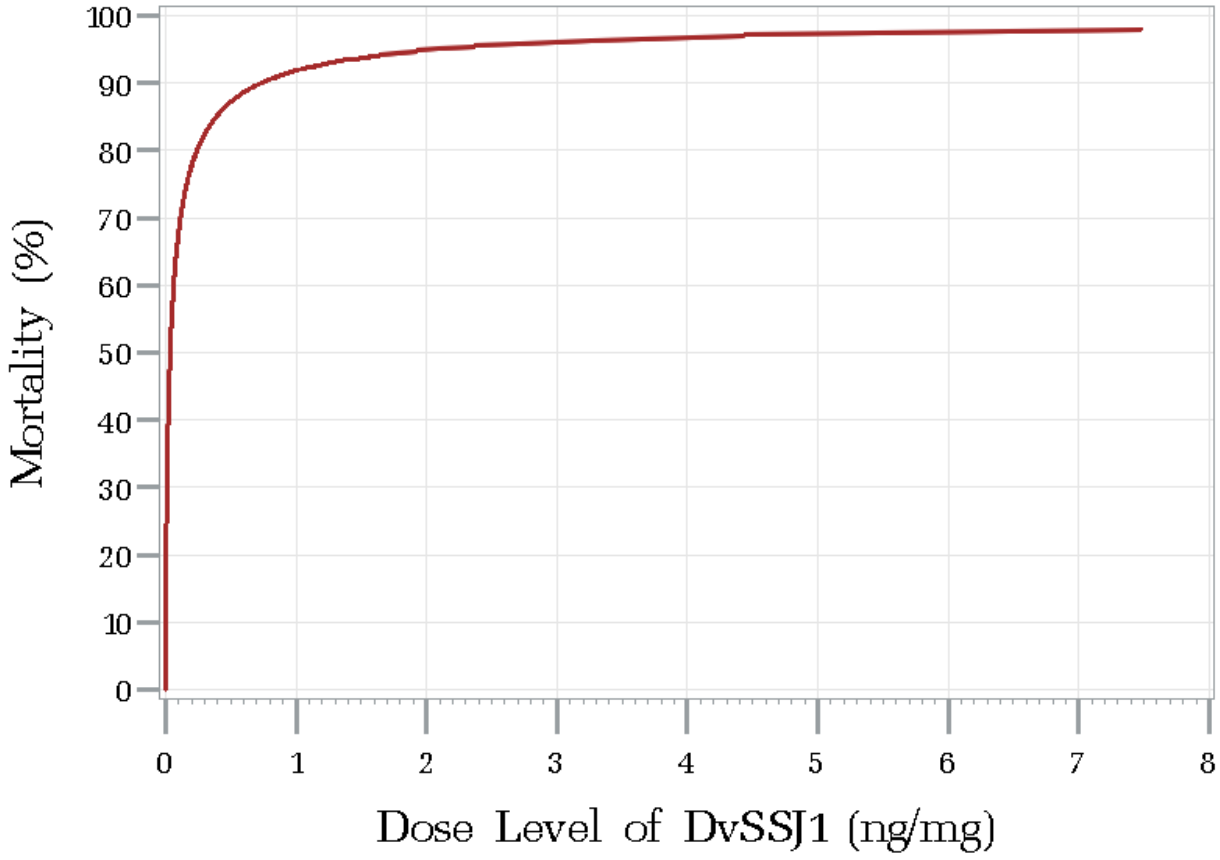


Figure 99. Meta-analysis Dose-response Curve for Bioassays with DvSSJ1_210 dsRNA Reported in D1. Evaluation of the Biological Response of Western Corn Rootworm Fed Artificial Diets Containing DvSSJ1_210 double stranded RNA.

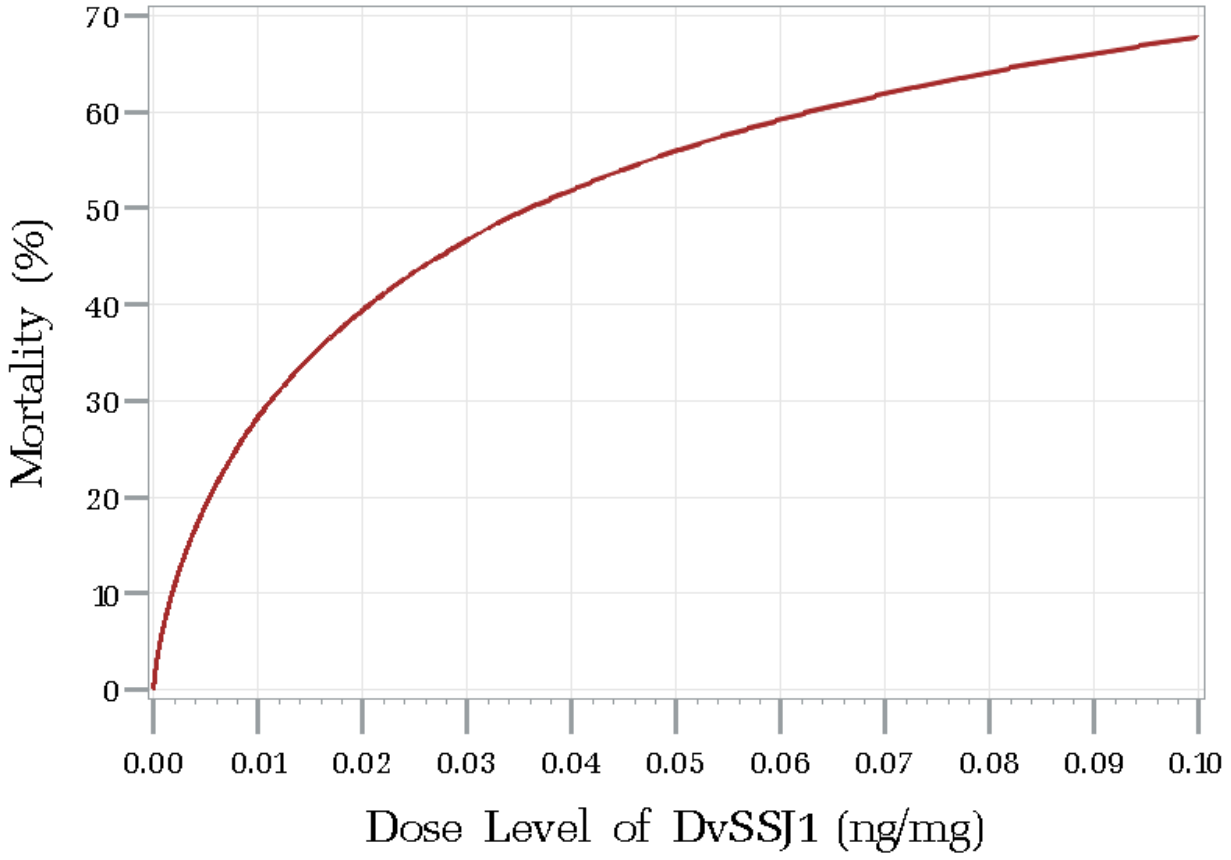


Figure 100. Meta-analysis Dose-response Curve for Bioassays with DvSSJ1_210 dsRNA Showing Greater Resolution of the Estimated Mortality Between 0 and 0.10 ng/mg.

Table 237. IPD072Aa Point Estimates, SEs, and 95% Confidence Intervals of Slope and Intercept for Individual Bioassays and the Overall Mean.

Bioassay	LC_{50}			Slope		
	Estimate	SE	95% Confidence Interval	Estimate	SE	95% Confidence Interval
1	16	2.8	11 - 21	5.3	1.5	2.3 - 8.2
2	16	3.1	10 - 22	7.8	3.5	0.91 - 15
3	12	1.8	8.7 - 16	7.5	1.8	3.9 - 11
Overall	14	1.5	11 - 17	6.3	1.1	4.2 - 8.4

Note: Lethal concentration (LC); standard error (SE).

Table 238. DvSSJ1_210 Point Estimates, SEs, and 95% Confidence Intervals of Slope and Intercept for Individual Bioassays and the Overall Mean.

Bioassay	LC_{50}			Slope		
	Estimate	SE	95% Confidence Interval	Estimate	SE	95% Confidence Interval
1	0.045	0.019	0.0064 - 0.083	1.8	0.36	1.1 - 2.5
2	0.042	0.027	0 - 0.096	4.7	3.1	-1.4 - 11
5	0.084	0.033	0.019 - 0.15	2.8	0.95	0.97 - 4.7
6	0.011	0.0077	0 - 0.026	1.3	0.34	0.61 - 2.0
Overall	0.036	0.015	0.0066 - 0.065	1.7	0.29	1.1 - 2.3

Note: Lethal concentration (LC); standard error (SE).

I.2. IPD072Aa 14 day LC50 with WCR

Experimental Design:

The purpose of this experiment was to assess the biological response of western corn rootworm, *Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae (WCR) when exposed to a range of IPD072Aa concentrations for 14 days. Additionally, an LC10, 20, 30, 40, and 50 were estimated.

All dilutions were prepared with ultrapure water at nominal concentration

- Bioassay conditions:
    - 21°C, 65% RH, no light
    - 14 day bioassay
    - Refed every 3 or 4 days
    - 24-well plates
    - 30 replicates

Experimental Design:

Trt	Rep Size	ng IPD072Aa / mg diet (by wet weight of diet)
1	30	0
2	30	1.0
3	30	2.0
4	30	4.0
5	30	8.0
6	30	16.0
7	30	32.0
8	30	64.0

Conclusions:

This estimated LC_{50} was used to inform the study Using *Diabrotica virgifera virgifera* to Evaluate the Combined Potency of IPD072Aa Protein and DvSSJ1_210 dsRNA (Appendix I. Using Diabrotica virgifera virgifera to Evaluate the Combined Potency ofIPD072Aa Protein and DvSSJ1_210 double-stranded RNA)

Table 240 details estimated doses for use in this study.

Table 239. The Point Estimates, SEs, and 95% Confidence Intervals of LC50s for Individual Bioassays and the Overall Mean.

Bioassay	LC_{50}		
	Estimate	SE	95% Confidence Interval
1	16	2.8	11 - 21
2	16	3.1	10 - 22
3	12	1.8	8.7 - 16
Overall	14	1.5	11 - 17

Table 240. Suggested WCR Dose for LC10, LC20, LC30, LC40 and LC50.

LC	Suggested Dose
10	6.30
20	8.46
30	10.3
40	12.1

Appendix J. Dissipation of DvSSJ1 210bp double-stranded RNA Incorporated in Loam, Sandy Clay Loam, and Silt Loam Soils

Test Substance

The test substance consisted of RNA oligonucleotide DvSSJ1_210 dsRNA. Stability was certified under -80 °C freezer unit storage condition.

Carrier (Bioassay)

The carrier for the bioassays was an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Test System

The test system in this study consisted of three soil types as described in Table 241.

Table 241. Physicochemical Characteristics of Soils

Parameter	Soil Characteristics		
USDA Textural Class	Loam	Sandy Loam	Silt Loam
Soil Sample ID	DU-L-PF	MSL-PF	Iowa
% Sand	48	64	20
% Silt	30	18	60
% Clay	22	18	20
Bulk Density (g/cm^3)	0.80	1.04	0.98
Cation Exchange Capacity (meq./100 g)	20.3	16.5	11.8
% Organic Matter[a]	13.2	2.8	3.6

Note: Soil classified as sandy loam by the USDA is referred to in this report as sandy clay loam soil.
[a] Determined using the Walkley Black method (Walkley and Black, 1934).

Soils were obtained from AGVISE Laboratories, Inc. (Northwood, ND, USA) and selected based on their representation of soil types likely to occur in the agroecosystem and their varied physicochemical properties.

Study Design

This study used three soil types (Table 241) spiked with DvSSJ1_210 dsRNA to estimate the dissipation of DvSSJ1_210 dsRNA over time. QuantiGene analysis was used to evaluate dissipation of DvSSJ1_210 dsRNA for each soil type. Insect bioassays using neonate *Diabrotica virgifera virgifera* (western corn rootworm; Coleoptera: Chrysomelidae), an insect sensitive to DvSSJ1_210 dsRNA, were used to evaluate the loss of insecticidal activity of DvSSJ1_210 dsRNA in each soil type.

Bias in sample collection and QuantiGene analysis was controlled through the randomizations of samples for each time point, replicate testing, appropriate assay controls, and pre-determined data acceptance criteria. Bias in the WCR bioassays was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Preparation of the DvSSJ1_210 dsRNA Soil Dosing Solution

The test substance was allowed to thaw at room temperature. A DvSSJ1_210 dsRNA soil dosing solution was prepared by diluting the test substance in RNase-free water to a target concentration of 15.26 µg/ml. A sub-sample of the soil dosing solution was collected and diluted at a ratio of 1:30.8 in QuantiGene Homogenizing Solutions (QHS; *i.e.* 10 µl dosing solution:308 µl QHS). The sample was stored frozen (-80°C freezer unit) until concentration verification by QuantiGene analysis as described in Section D and appendix A. The soil dosing solution was maintained at room temperature during preparation and use.

Soil Preparation

Processing

Aliquots of the three soil types were weighed into numbered tubes to a target weight of 750 mg (± 5%), for a total of 70 tubes per soil type. The tubes were loosely sealed with caps and stored in tube racks separated by soil type in a dark environmental chamber set at 20 °C and 80% relative humidity for approximately 48 hours prior to spiking with dosing solution.

Dosing of Soil Samples

Soil samples were removed from the environmental chamber. DvSSJ1_210 dsRNA soil dosing solution (120 µl) or RNase-free water (120 µl) were added to the soil aliquots, creating 45 DvSSJ1_210 dsRNA samples and 25 control samples for each soil type. Due to slight differences in moisture content of the three soil types, the nominal concentrations of DvSSJ1_210 dsRNA (dry weight) within each soil sample type were as follows: 3.27 ng/mg (loam); 2.83 ng/mg (sandy clay loam); and 3.05 ng/mg (silt loam). Each tube was then sealed with parafilm and poked with a target of two pinholes. Two DvSSJ1_210 dsRNA spiked samples (designated as Time Zero samples) and one control sample from each soil type were collected for QuantiGene analysis, as described in Section D. In addition, for the initial insect bioassay, one spiked and one control sample from each soil type were collected for incorporation into diet for Day 0 feeding, as described in Section E. Remaining samples were returned to the environmental chamber under the same incubation conditions until use, as applicable.

QuantiGene Analysis

Immediately following soil dosing and at several time points after, and at intervals throughout the six bioassays, two randomly selected (with the exception of Time Zero) aliquots of each DvSSJ1_210 dsRNA spiked soil type were collected and extracted for QuantiGene analysis (Table 2 below and as described in the Analytical Phase section below).

***WCR* Bioassays**

WCR bioassays were initiated on the day soils were spiked (Day 0) and at subsequent 7-day intervals (initiated with Day 7 and 14 soil samples) for the first three bioassays (Bioassay 1-3) followed by three additional bioassays at 14-day intervals (initiated with Day 28, 42 and 56 soil samples) until the observed *WCR* mortality was ≤ 30% for the diets incorporated with DvSSJ1_210 dsRNA spiked soils. This resulted in a total of 6 bioassays (Table 242).

Table 242. Collection of Soil Samples for QuantiGene and Bioassay Assessments

Day	**0[a]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
QuantiGene Analysis	X	X		X											
Bioassay 1	X				X			X				X			
Day	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
QuantiGene Analysis	X														
Bioassay 2	X				X			X				X			
Day	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
QuantiGene Analysis	X										X				
Bioassay 3	X				X			X				X			
Day	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
QuantiGene Analysis					X										
Bioassay 4	X[b]			X[b]				X[b]				X[b]			
Day	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**
QuantiGene Analysis	X														
Bioassay 5	X[c]				X[c]			X[c]				X[c]			
Day	**56**	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**
QuantiGene Analysis	X[c]														X[c]
Bioassay 6	X[c]				X[c]			X[c]				X[c]			

Note: The 14-day bioassays were conducted consecutively, with bioassays 1, 2, and 3 at 7-day intervals and bioassays 4, 5, and 6 at 14-day intervals.

[a] Day soils were spiked with DvSSJ1_210 dsRNA.

[b] Only loam and silt loam soil samples were collected.

[c] Only loam soil samples were collected.

For each bioassay, aliquots of each soil type were incorporated into artificial diet (20% by dry weight) and *WCR* larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Loam soil control spiked with RNase-free water
- Treatment 2: Sandy clay loam soil control spiked with RNase-free water

- Treatment 3: Silt loam soil control spiked with RNase-free water
- Treatment 4: Loam soil spiked with DvSSJ1_210 dsRNA
- Treatment 5: Sandy clay loam soil spiked with DvSSJ1_210 dsRNA
- Treatment 6: Silt loam soil spiked with DvSSJ1_210 dsRNA

The bioassay acceptability criterion indicated the bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 30% for the bioassay controls containing water-spiked soils.

Data Analysis

Mortality data for each bioassay were summarized by calculation of the number of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100. Weight data were summarized as means, standard deviations, and ranges.

Results and Discussion

QuantiGene analysis showed approximately 80% loss of DvSSJ1_210 dsRNA within 24 hours across all soil types (**Figure 101**; **Figure 102**; **Figure 103**; and **Table 243**; **Table 244**; **Table 245**). Continued dissipation of DvSSJ1_210 dsRNA was observed at each of the subsequent sample periods. The concentration of the DvSSJ1_210 dsRNA soil dosing solution was also verified (103% of expected value; **Table 246**)

Each WCR bioassay met the acceptability criterion as the combined dead and missing WCR count did not exceed 30% in any of the bioassay control diets with water-spiked soils.

In total, 6 bioassays were conducted using soil samples ranging from 0 to 67 days old. In the first bioassay initiated with Day 0 soil, the mortality for WCR provided diets prepared with DvSSJ1_210 dsRNA-spiked soils was 100%, 79.3%, and 73.1% for the loam, sandy clay loam, and silt loam soil groups, respectively (**Table 247**). In the second bioassay initiated with Day 7 soil, the mortality for WCR provided diets prepared with DvSSJ1_210 dsRNA-spiked soils was 86.2%, 73.3%, and 73.3% for the loam, sandy clay loam, and silt loam soil groups, respectively (**Table 248**). In the third bioassay initiated with Day 14 soil, the mortality for WCR provided diets prepared with DvSSJ1_210 dsRNA-spiked soils was 46.7%, 23.3%, and 33.3% for the loam, sandy clay loam, and silt loam soil groups, respectively (**Table 249**). As the observed mortality was ≤ 30% for the sandy clay loam DvSSJ1_210 dsRNA-spiked soil following the third bioassay, no additional bioassays with this soil were conducted. In the fourth bioassay initiated with Day 28 soil, the mortality for WCR provided diets prepared with DvSSJ1_210 dsRNA-spiked soils was

56.7% and 30.0% for the loam and silt loam soil groups, respectively (**Table 250**). As the observed mortality was ≤ 30% for the silt loam DvSSJ1_210 dsRNA-spiked soil following the fourth bioassay, no additional bioassays with this soil were conducted. In the fifth bioassay initiated with Day 42 soil and the sixth bioassay initiated with Day 56 soil, the mortality for WCR provided diets prepared with DvSSJ1_210 dsRNA-spiked soil was 43.3% and 36.7% respectively (**Table 251**; **Table 252**).

Weight data for the six bioassays were summarized as means, standard deviations, and ranges and are presented in **Table 247**, **Table 248**, **Table 249**, **Table 250**, **Table 251**, and **Table 252**.

Conclusion

Dissipation of the DvSSJ1_210 dsRNA was observed via QuantiGene analysis with approximately 80% reduction within 24 hours in all three soil types. Bioassays with WCR showed loss of bioactivity within 30 days in sandy clay loam and silt loam soils. Bioactivity in the loam soil showed a reduction in potency from day 0 to day 55. Based on the demonstrated dissipation of the DvSSJ1_210 dsRNA via QuantiGene analysis and the loss of insecticidal activity in a variety of soil types, the DvSSJ1 dsRNA is unlikely to persist or accumulate in soil.

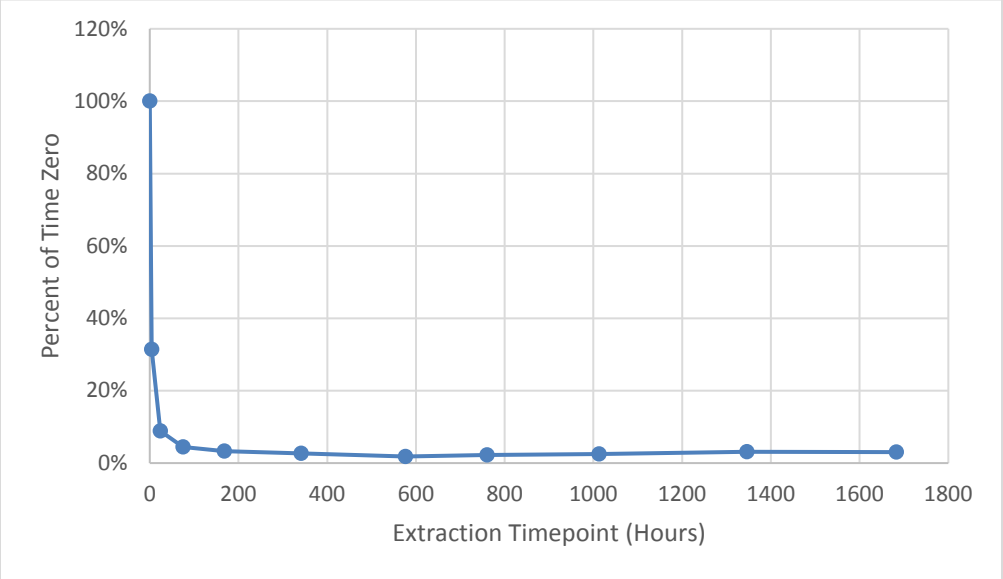


Figure 101. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Loam Soil as a Percentage of Time Zero

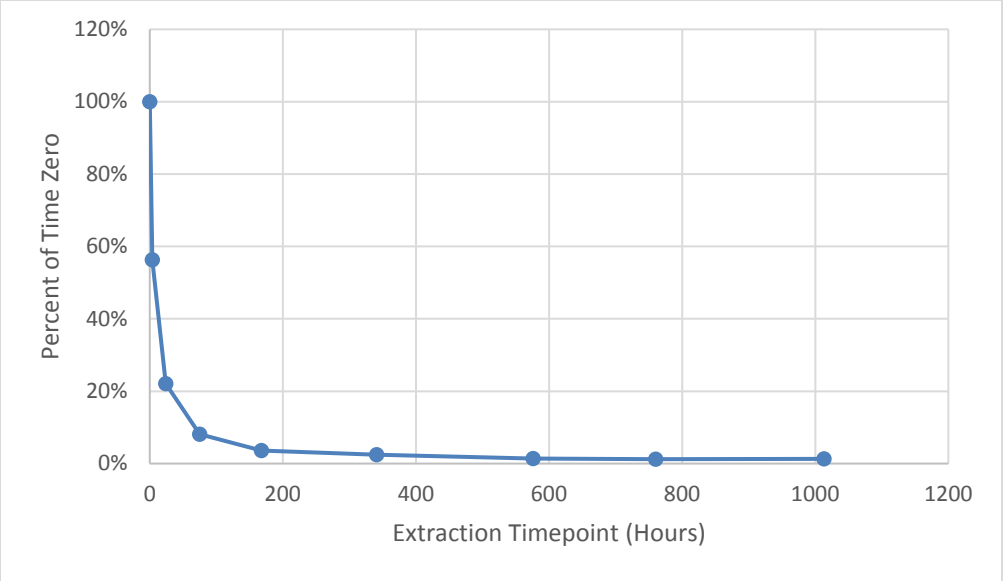


Figure 102. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Sandy Clay Loam Soil as a Percentage of Time Zero

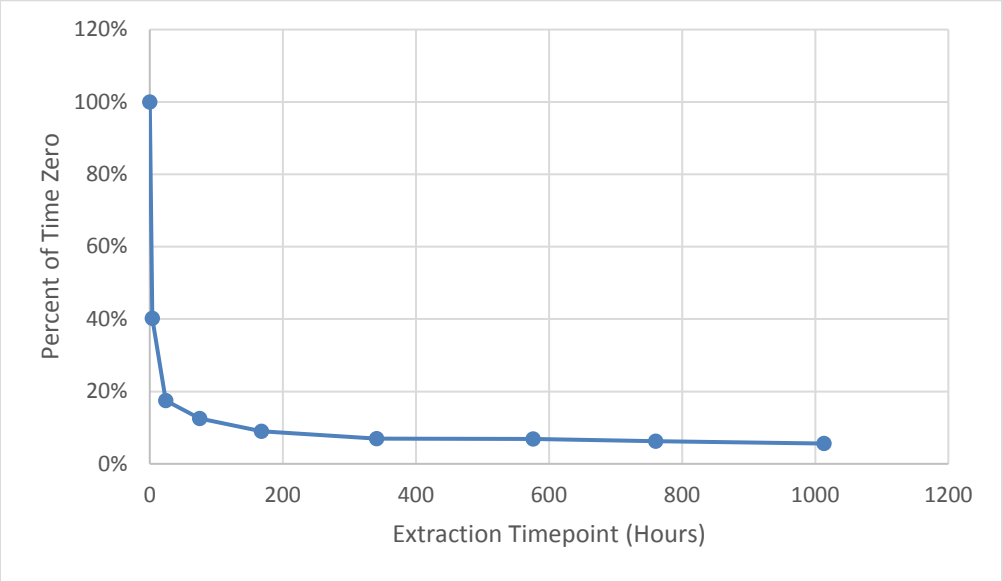


Figure 103. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Silt Loam Soil as a Percentage of Time Zero

Table 243. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Loam Soil

Timepoint (hours)	0	4	24	75	168	341	576	760	1013	1346	1683
Percent of Time Zero	100%	31%	9%	4%	3%	3%	2%	2%	3%	3%	3%

Table 244. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Sandy Clay Loam Soil

Timepoint (hours)	0	4	24	75	168	341	576	760	1013
Percent of Time Zero	100%	56%	22%	8%	4%	2%	1%	1%	1%

Table 245. QuantiGene Analysis of DvSSJ1_210 dsRNA Dissipation in Silt Loam Soil

Timepoint (hours)	0	4	24	75	168	341	576	760	1013
Percent of Time Zero	100%	40%	18%	13%	9%	7%	7%	6%	6%

Table 246. Concentration Verification of DvSSJ1_210 dsRNA Soil Dosing Solution by QuantiGene Analysis

Sample	Percent of expected (%)
Plate 1	103
Plate 2	104
Plate 3	102
Mean	103

Note: Concentrations within 70-130% of the expected value were considered verified.

Table 247. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 0 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Control Loam Soil	28[a]	7.14	26	0.681 ± 0.397	0.2 - 1.8
2	Control Sandy Clay Loam Soil	30	3.33	29	0.738 ± 0.506	0.2 - 2.4
3	Control Silt Loam Soil	30	0.00	30	0.980 ± 0.639	0.1 - 2.4
4	Loam Soil Spiked with DvSSJ1_210 dsRNA	30	100	0	NA	NA
5	Sandy Clay Loam Soil Spiked with DvSSJ1_210 dsRNA	29[a]	79.3	6	0.300 ± 0.245	0.1 - 0.7
6	Silt Loam Soil Spiked with DvSSJ1_210 dsRNA	26[a]	73.1	7	0.357 ± 0.162	0.1 - 0.5

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% soil incorporated into dry carrier (by carrier dry weight). Treatments 1, 2, and 3 contained soil spiked with RNase-free water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 248. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 7 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Control Loam Soil	30	10.0	27	0.396 ± 0.281	0.1 - 1.3
2	Control Sandy Clay Loam Soil	30	10.0	27	0.511 ± 0.365	0.1 - 1.6
3	Control Silt Loam Soil	30	3.33	29	0.741 ± 0.628	0.1 - 2.3
4	Loam Soil Spiked with DvSSJ1_210 dsRNA	29[a]	86.2	4	0.250 ± 0.173	0.1 - 0.5
5	Sandy Clay Loam Soil Spiked with DvSSJ1_210 dsRNA	30	73.3	8	0.388 ± 0.223	0.1 - 0.8
6	Silt Loam Soil Spiked with DvSSJ1_210 dsRNA	30	73.3	8	0.250 ± 0.0926	0.1 - 0.4

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% soil incorporated into dry carrier (by carrier dry weight). Treatments 1, 2, and 3 contained soil spiked with RNase-free water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 249. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 14 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Control Loam Soil	29[a]	6.90	27	0.722 ± 0.459	0.2 - 1.9
2	Control Sandy Clay Loam Soil	30	13.3	26	1.11 ± 0.738	0.2 - 2.6
3	Control Silt Loam Soil	30	3.33	29	1.09 ± 0.732	0.1 - 2.7
4	Loam Soil Spiked with DvSSJ1_210 dsRNA	30	46.7	16	0.238 ± 0.0885	0.1 - 0.4
5	Sandy Clay Loam Soil Spiked with DvSSJ1_210 dsRNA	30	23.3	23	0.309 ± 0.156	0.1 - 0.7
6	Silt Loam Soil Spiked with DvSSJ1_210 dsRNA	30	33.3	20	0.335 ± 0.246	0.1 - 1.1

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% soil incorporated into dry carrier (by carrier dry weight). Treatments 1, 2, and 3 contained soil spiked with RNase-free water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 250. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 28 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Control Loam Soil	30	3.33	29	0.921 ± 0.768	0.1 - 2.7
2	Control Sandy Clay Loam Soil	NA[a]	NA	NA	NA	NA
3	Control Silt Loam Soil	30	0.00	30	1.06 ± 0.683	0.3 - 2.3
4	Loam Soil Spiked with DvSSJ1_210 dsRNA	30	56.7	13	0.246 ± 0.133	0.1 - 0.5
5	Sandy Clay Loam Soil Spiked with DvSSJ1_210 dsRNA	NA	NA	NA	NA	NA
6	Silt Loam Soil Spiked with DvSSJ1_210 dsRNA	30	30.0	21	0.357 ± 0.186	0.1 - 0.8

Note: Treatments 1, 3, 4, and 6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% soil incorporated into dry carrier (by carrier dry weight). Treatments 1, and 3 contained soil spiked with RNase-free water.

[a] Not applicable (NA); this treatment was not included in this bioassay as the observed *WCR* mortality in the previous bioassay was ≤ 30% for this soil type spiked with DvSSJ1_210 dsRNA.

Table 251. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 42 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Control Loam Soil	29[a]	3.45	28	0.989 ± 0.702	0.3 - 2.6
2	Control Sandy Clay Loam Soil	NA[b]	NA	NA	NA	NA
3	Control Silt Loam Soil	NA	NA	NA	NA	NA
4	Loam Soil Spiked with DvSSJ1_210 dsRNA	30	43.3	17	0.265 ± 0.141	0.1 - 0.6
5	Sandy Clay Loam Soil Spiked with DvSSJ1_210 dsRNA	NA	NA	NA	NA	NA
6	Silt Loam Soil Spiked with DvSSJ1_210 dsRNA	NA	NA	NA	NA	NA

Note: Treatments 1 and 4 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% soil incorporated into dry carrier (by carrier dry weight). Treatment 1 contained soil spiked with RNase-free water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Not applicable (NA); this treatment was not included in this bioassay as the observed *WCR* mortality in the previous bioassay was ≤ 30% for this soil type spiked with DvSSJ1_210 dsRNA.

Table 252. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 56 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Control Loam Soil	29[a]	3.45	28	0.836 ± 0.613	0.2 - 2.3
2	Control Sandy Clay Loam Soil	NA[b]	NA	NA	NA	NA
3	Control Silt Loam Soil	NA	NA	NA	NA	NA
4	Loam Soil Spiked with DvSSJ1_210 dsRNA	30	36.7	19	0.295 ± 0.118	0.1 - 0.6
5	Sandy Clay Loam Soil Spiked with DvSSJ1_210 dsRNA	NA	NA	NA	NA	NA
6	Silt Loam Soil Spiked with DvSSJ1_210 dsRNA	NA	NA	NA	NA	NA

Note: Treatments 1 and 4 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% soil incorporated into dry carrier (by carrier dry weight). Treatment 1 contained soil spiked with RNase-free water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

[b] Not applicable (NA); this treatment was not included in this bioassay as the observed *WCR* mortality in the previous bioassay was ≤ 30% for this soil type spiked with DvSSJ1_210 dsRNA.

J.1. Analytical Phase

QuantiGene analysis was used to assess the dissipation of DvSSJ1_210 dsRNA in loam, sandy clay loam, and silt loam soils over time. The QuantiGene Plex Assay is a multiplexed gene expression quantification assay which combines branched DNA signal amplification and magnetic multi-analyte profiling bead technologies to enable the measurement of multiple RNA transcripts/targets simultaneously.

Sample Collection

At the time of soil spiking (Time Zero) and at intervals through Day 42, two randomly selected samples of loam, sandy clay loam, and silt loam soil spiked with DvSSJ1_210 dsRNA were collected in preparation for QuantiGene analysis. On Day 56 (two randomly selected samples) and Day 70 (one sample), samples of loam soil spiked with DvSSJ1_210 dsRNA were collected in preparation for QuantiGene analysis. At Time Zero, a control soil sample from each soil type was also collected for QuantiGene analysis.

Sample Extraction

Soil samples were extracted at room temperature. QuantiGene Homogenizing Solution (QHS; 3820 µl) was added to each tube containing soil and samples were vortexed continuously for four minutes, and centrifuged. The supernatants were transferred to fresh tubes and centrifuged again and the final supernatants (*i.e.*, sample homogenates) were stored frozen (-80 °C freezer unit), if applicable, until QuantiGene analysis. The Time Zero control soil was extracted but not analyzed.

QuantiGene Analysis

The DvSSJ1 QuantiGene method utilized magnetic beads specific to the target to measure the amount of DvSSJ1_210 dsRNA in samples. Prior to analysis, samples of soil dosing solution and extracted soil samples were removed from the freezer (if applicable), warmed, vortexed, and then diluted as necessary in QHS. Standards and samples (both typically analyzed in triplicate wells) were first denatured and annealed in a 96-well PCR plate with a sequence-specific probe set that included Capture Extenders (CE), Label Extenders (LE), and Blocking Probes. The plate containing target-probe complex in each well was then transferred to a hybridization plate, where it was incubated overnight with magnetic beads that hybridize to the CE probes. Following incubation, a magnetic separation device was used to wash unbound substances from the plate. Signal amplification was amplified on the LE probes by sequential incubations with pre-amplifier, amplifier, and label probes, with each incubation followed by a wash step to remove unbound substances. Each well was then incubated with the fluorescent protein streptavidin phycoerythrin (SAPE), and then washed. The SAPE generated a signal that was

proportional to the amount of DvSSJ1_210 dsRNA present in the reaction. The median fluorescence intensity (MFI) of each well was then determined using a MAGPIX Multiplex Reader running xPonent v 4.2 software.

Calculations

A standard curve was prepared by diluting the test substance in QHS. A standard curve was loaded to all plates and was linear at 0.03125-0.25 pg/well DvSSJ1_210 dsRNA. A QHS buffer blank was also loaded to each plate and the average of the buffer blank was subtracted from all wells on the plate. The concentration of DvSSJ1_210 dsRNA in the soil dosing solution was interpolated using the standard curve.

For relative comparisons (*i.e.*, dissipation over time), the background-corrected MFI was used for calculations.

Verification of DvSSJ1_210 dsRNA Concentration in the Soil Dosing Solution

The concentration of DvSSJ1_210 dsRNA in soil dosing solution was determined by averaging the results from three separate analyses and comparing them to the expected value. The concentration was considered verified if the results fell within 70-130% of the expected value.

Assessment of DvSSJ1_210 dsRNA Dissipation in Soil Samples

The dissipation of DvSSJ1_210 dsRNA in soil samples was calculated for each time point as a percentage of Time 0 mean MFI by dividing the mean MFI of two replicates per time point by the mean Time 0 MFI result, multiplied by 100.

J.2. Bioassay Details

Treatments were arranged in a generalized randomized block design with a total of 3, 5, or 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3, 6, or 10 replicates from each treatment, dependent on the number of blocks. Each treatment was fed to a target of 30 *Diabrotica virgifera virgifera* (western corn rootworm; Coleoptera: Chrysomelidae) individuals. Bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark, with the exception of a 21-hour period during bioassays 1 and 2 when the lights were inadvertently turned on. The 14-day bioassays were conducted consecutively, with bioassays 1, 2, and 3 at 7-day intervals and bioassays 4, 5, and 6 at 14-day intervals. Larvae were refed every 3 or 4 days of each bioassay with soil aliquots that had been incubated an additional 3 or 4 days after the previous feeding with some overlap for the first three bioassays (*e.g.,* the first bioassay used soils incubated for 0, 4, 7, and 11 days; the second bioassay used soils incubated for 7, 11, 14, and 18 days, as described in Table 2). Bioassays were complete after 14 days of feeding. Mortality was assessed and surviving organisms were individually weighed. Mortality results were used to demonstrate the loss of insecticidal activity for the DvSSJ1_210 dsRNA in each soil type.

Diet Treatment Preparation

On each day of feeding, samples from each soil type were collected and used to create artificial diet to feed *WCR*. On each day of diet preparation, one randomly selected aliquot each of the DvSSJ1_210 dsRNA-spiked soil and control soil for each soil type were removed from the environmental chamber and mixed with carrier at approximately 20% soil by dry weight of carrier. RNase-free water was added to the dry soil-carrier mix at a 2.51:1 ratio (*i.e.*, 2.51 ml water to 1 g soil-carrier mix), generating Treatments 1-6, as applicable.

WCR Bioassays

WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. A separate batch of WCR eggs for each bioassay was incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassays within 24 hours of hatching.

On Day 0 of each respective bioassay, approximately 300 µl (*i.e.*, 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One WCR neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. Bioassays were conducted for 14 days in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark, with the exception of a 21-hour period during bioassays 1 and 2 when the lights were inadvertently turned on. On Days 3 or 4, 7, and 11 of

each respective bioassay, new plates were prepared with fresh diet as described for Day 0, living *WCR* larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 14 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

Appendix K. Dissipation of IPD072Aa Protein Incorporated in Loam, Sandy Clay Loam, and Silt Loam Soils

Test Substance

The test substance consisted of IPD072Aa protein solubilized from a lyophilized powder (lot number PCF-0040; molecular weight ~10 kDa). Protein stability was certified under refrigerated storage condition.

Carrier (Bioassay)

The carrier for the bioassay is an artificial diet consisting primarily of Stonefly Heliothis diet prepared by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

Test System

The test system in this study consisted of three soil types as described in Table 253.

Table 253. Physiochemical Characteristics of Soil

Parameter	Soil Characteristics		
USDA Textural Class	Loam	Sandy Clay Loam	Silt Loam
Soil Sample ID	DU-L-PF	MSL-PF	Iowa
% Sand	35	61	15
% Silt	42	16	64
% Clay	23	23	21
Bulk Density (g/cm^3)	0.77	1.00	0.92
Cation Exchange Capacity (meq./100 g)	21.3	15.1	12.4
% Organic Matter[a]	11.5	3.1	4.8

[a] Determined using the Walkley Black method (Walkley and Black, 1934).

Soils were obtained from AGVISE Laboratories, Inc. (Northwood, ND, USA) and selected based on their representation of soil types likely to occur in the agroecosystem and their varied physicochemical properties.

Study Design

This study used three soil types (Table 253) spiked with IPD072Aa protein to estimate the dissipation of IPD072Aa protein over time. Qualitative western blot analyses were used to visually evaluate dissipation of immunoreactive IPD072Aa protein for each soil type. Insect bioassays using neonate *Diabrotica virgifera virgifera* (western corn rootworm; Coleoptera: Chrysomelidae), an insect sensitive to IPD072Aa protein, were used to evaluate the loss of insecticidal activity of IPD072Aa protein in each soil type.

Bias in the western blot analysis portion of the study was controlled through the randomization of samples for each time point and the inclusion of a molecular weight standard and IPD072Aa stock solution sample on each gel. Bias in the *WCR* bioassays was controlled through the randomization of treatments within blocks and the use of one or more control diets.

Preparation and Storage of the IPD072Aa Protein Stock Solution

Lyophilized powder containing IPD072Aa protein was solubilized in chilled ultrapure water and clarified by centrifugation to create the IPD072Aa protein test substance stock solution. The concentration of IPD072Aa protein in the stock solution was determined to be 26.7 mg/ml by absorbance measurement at A280 using the NanoDrop 2000c Spectrophotometer. A280 measurements were taken on the day of preparation using NanoDrop settings of sample type other protein (E & MW), and theoretical IPD072Aa protein values (ε/1000=13.94 and molecular weight of 9.55 kDa), based on the protein sequence. The IPD072Aa protein stock solution was stored refrigerated (2-8 °C) or on wet ice until use.

Soil Preparation

Processing

Aliquots of the three soil types were weighed into numbered microtubes to a target weight of 250 mg (± 5%), for a total of 90 microtubes per soil type. The microtubes were sealed with caps containing a pinhead-sized hole and stored in tube racks separated by soil type in a dark environmental chamber set at 20 °C and 80% relative humidity for approximately 42 hours prior to spiking.

Dosing of Soil Samples

Ninety microtubes of each soil type were removed from the environmental chamber and any visible plant biomass was removed. Forty µl of IPD072Aa protein stock solution or 40 µl of ultrapure water were added to the soil aliquots, creating 57 IPD072Aa protein samples and 33 control samples for each soil type. Due to slight differences in moisture content of the three soil types, the nominal concentration of IPD072Aa protein (dry weight) within each soil sample type were as follows: 5,652 ng/mg (loam); 5,037 ng/mg (sandy clay loam); and 5,267 ng/mg (silt loam). Each microtube was then sealed with caps containing a pinhead-sized hole. Three IPD072Aa spiked samples (designated as Day 0 samples) from each soil type were collected for the western blot analysis. For the initial insect bioassay, an additional 3 spiked and 3 control samples from each soil type were collected for incorporation into diet. All other samples were placed back in a dark environmental chamber set at 20 °C and 80% relative humidity until use.

Western Blot Analysis

On the day soils were dosed (Day 0) and days 1, 2, 3, 4, 7, and 14, three randomly selected aliquots of each IPD072Aa spiked soil type were collected and extracted for western blot analysis (Table 2), as described in the Analytical Phase section below.

WCR Bioassays

WCR bioassays were conducted on the day soils were spiked (Day 0) and at subsequent 7-day intervals until the observed WCR mortality was ≤ 30% for the diets with IPD072Aa protein spiked soils. This resulted in a total of 2 bioassays (Table 254). For each bioassay, aliquots of each soil type were incorporated into artificial diet (20% by dry weight) and WCR larvae were exposed via oral ingestion to one of the following six treatments:

Treatment 1: Loam soil spiked with IPD072Aa protein

Treatment 2: Loam soil control (spiked with ultrapure water)

Treatment 3: Sandy clay loam soil spiked with IPD072Aa protein

Treatment 4: Sandy clay loam soil control (spiked with ultrapure water)

Treatment 5: Silt loam soil spiked with IPD072Aa protein

Treatment 6: Silt loam soil control (spiked with ultrapure water)

Table 255 highlights IPD072Aa protein concentrations in each soil type and calculated concentrations for the initial diet provided on Day 0. Additional details regarding the *WCR* bioassays are provided in the Bioassay Details section below.

Table 254. Collection of Soil Samples for Western Blot and Bioassay Assessments

Assessment Method	Day														
	0[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Western Blots	X	X	X	X	X			X							X
Bioassay 1 Soil Collections	X				X										
Bioassay 2 Soil Collections								X				X			

[a] Day soils were spiked with IPD072Aa protein.

Table 255. Initial Soil Concentrations and Resulting Calculated Day 0 Bioassay Concentrations of the IPD072Aa Protein

Soil	Initial Soil Concentration (ng/mg dry weight)	Bioassay Day 0 Diet Concentration (ng/mg dry weight)
Loam	5652	854.4
Sandy Clay Loam	5037	854.4
Silt Loam	5267	854.4

Data Analysis

Mortality data for each bioassay were summarized by calculation of dead larvae divided by the total number of observed larvae at the end of the bioassay multiplied by 100. Weight data were summarized as means, standard deviations, and ranges.

Results and Discussion

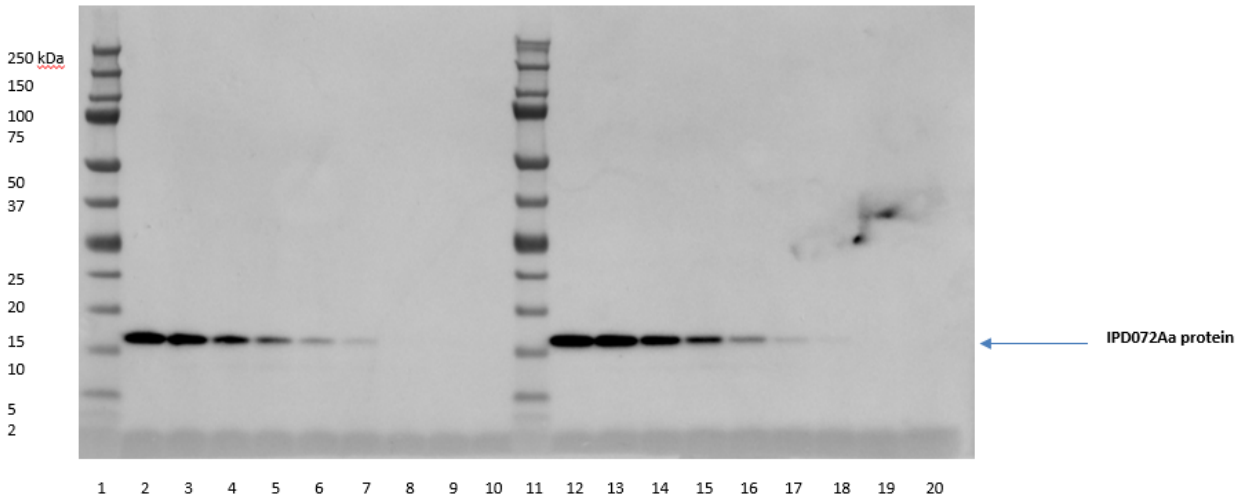
Dissipation of IPD072Aa protein in loam, sandy clay loam, and silt loam soil within 7 days was visually confirmed by western blot analysis (Figure 104; Figure 105; Figure 106). Differences in protein band intensities are not unexpected due to the qualitative nature of the western blot analysis used in this study to support conclusions as to IPD072Aa protein dissipation in soil. For the loam soil, no prominent IPD072Aa protein band was visible after Day 7. For the sandy clay loam and silt loam soils, no prominent IPD072Aa protein band was visible after Day 2.

Each *WCR* bioassay met the acceptability criterion as the combined dead and missing WCR count did not exceed 30% in any of the bioassay control diets with water-spiked soils.

In total, 2 bioassays were conducted. In the first bioassay, the mortality for WCR provided diets prepared with IPD072Aa protein-spiked soils (Day 0 and Day 4) was 67.9%, 58.6%, and 50.0% for the loam, sandy clay loam, and silt loam soil groups, respectively (Table 256). In the second bioassay, mortality for WCR provided diets prepared with IPD072Aa protein-spiked soils (Day 7 and Day 11) was 0%, 0%, and 6.67% for the loam, sandy clay loam, and silt loam soil groups, respectively (Table 257). As the observed mortality was below 30% for all three IPD072Aa protein-spiked soils following the second bioassay, no additional bioassays were conducted. Weight data for both bioassays were summarized as means, standard deviations, and ranges and are presented in Table 256 and Table 257.

Conclusion

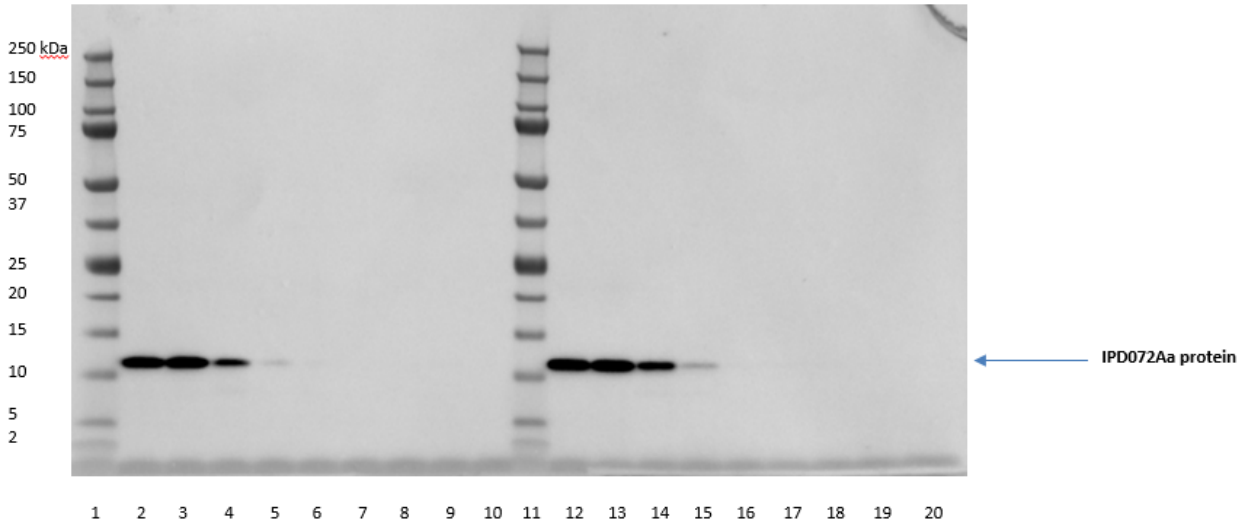
Collectively, visual confirmation using western blot analyses and mortality results from WCR bioassays both support rapid dissipation of the IPD072Aa protein within 7 days across multiple soil types.



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Pre-stained Protein Molecular Weight Markers
2	IPD072Aa Protein Test Substance (10 ng)	12	IPD072Aa Protein Test Substance (10 ng)
3	Loam Soil Extract (Replicate 1), Day 0	13	Loam Soil Extract (Replicate 2), Day 0
4	Loam Soil Extract (Replicate 1), Day 1	14	Loam Soil Extract (Replicate 2), Day 1
5	Loam Soil Extract (Replicate 1), Day 2	15	Loam Soil Extract (Replicate 2), Day 2
6	Loam Soil Extract (Replicate 1), Day 3	16	Loam Soil Extract (Replicate 2), Day 3
7	Loam Soil Extract (Replicate 1), Day 4	17	Loam Soil Extract (Replicate 2), Day 4
8	Loam Soil Extract (Replicate 1), Day 7	18	Loam Soil Extract (Replicate 2), Day 7
9	Loam Soil Extract (Replicate 1), Day 14	19	Loam Soil Extract (Replicate 2), Day 14
10	1X LDS/DTT Sample Buffer Blank	20	1X LDS/DTT Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the Day 0 spiked samples. Days 0-14 refer to the number of days for which spiked soil samples were incubated prior to analysis.

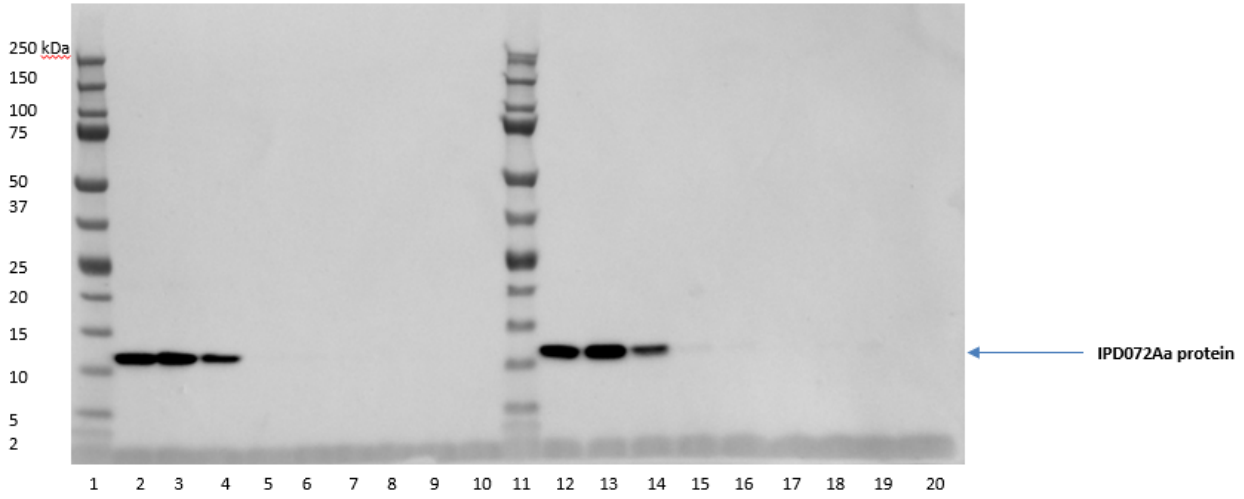
Figure 104. Western Blot Analysis of IPD072Aa Protein Dissipation in Loam Soil



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Pre-stained Protein Molecular Weight Markers
2	IPD072Aa Protein Test Substance (10 ng)	12	IPD072Aa Protein Test Substance (10 ng)
3	Sandy Clay Loam Soil Extract (Replicate 1), Day 0	13	Sandy Clay Loam Soil Extract (Replicate 2), Day 0
4	Sandy Clay Loam Soil Extract (Replicate 1), Day 1	14	Sandy Clay Loam Soil Extract (Replicate 2), Day 1
5	Sandy Clay Loam Soil Extract (Replicate 1), Day 2	15	Sandy Clay Loam Soil Extract (Replicate 2), Day 2
6	Sandy Clay Loam Soil Extract (Replicate 1), Day 3	16	Sandy Clay Loam Soil Extract (Replicate 2), Day 3
7	Sandy Clay Loam Soil Extract (Replicate 1), Day 4	17	Sandy Clay Loam Soil Extract (Replicate 2), Day 4
8	Sandy Clay Loam Soil Extract (Replicate 1), Day 7	18	Sandy Clay Loam Soil Extract (Replicate 2), Day 7
9	Sandy Clay Loam Soil Extract (Replicate 1), Day 14	19	Sandy Clay Loam Soil Extract (Replicate 2), Day 14
10	1X LDS/DTT Sample Buffer Blank	20	1X LDS/DTT Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the spiked samples. Days 0-14 refer to the number of days for which spiked soil samples were incubated prior to analysis.

Figure 105. Western Blot Analysis of IPD072Aa Protein Dissipation in Sandy Clay Loam Soil



Lane	Sample Identification	Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Markers	11	Pre-stained Protein Molecular Weight Markers
2	IPD072Aa Protein Test Substance (10 ng)	12	IPD072Aa Protein Test Substance (10 ng)
3	Silt Loam Soil Extract (Replicate 1), Day 0	13	Silt Loam Soil Extract (Replicate 2), Day 0
4	Silt Loam Soil Extract (Replicate 1), Day 1	14	Silt Loam Soil Extract (Replicate 2), Day 1
5	Silt Loam Soil Extract (Replicate 1), Day 2	15	Silt Loam Soil Extract (Replicate 2), Day 2
6	Silt Loam Soil Extract (Replicate 1), Day 3	16	Silt Loam Soil Extract (Replicate 2), Day 3
7	Silt Loam Soil Extract (Replicate 1), Day 4	17	Silt Loam Soil Extract (Replicate 2), Day 4
8	Silt Loam Soil Extract (Replicate 1), Day 7	18	Silt Loam Soil Extract (Replicate 2), Day 7
9	Silt Loam Soil Extract (Replicate 1), Day 14	19	Silt Loam Soil Extract (Replicate 2), Day 14
10	1X LDS/DTT Sample Buffer Blank	20	1X LDS/DTT Sample Buffer Blank

Note: Nanogram (ng). Molecular weight markers (measured in kilodaltons kDa) were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight (~10 kDa). The test substance consisted of IPD072Aa protein solubilized in ultrapure water. Samples were loaded to a target of 10 ng based on the nominal concentration of IPD072Aa protein in the spiked samples. Days 0-14 refer to the number of days for which spiked soil samples were incubated prior to analysis.

Figure 106. Western Blot Analysis of IPD072Aa Protein Dissipation in Silt Loam Soil

Table 256. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 0 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Loam Soil Spiked with IPD072Aa Protein	28[a]	67.9	9	0.0889 ± 0.0601	0.0 - 0.2
2	Control Loam Soil	30	0	30	0.350 ± 0.117	0.1 - 0.6
3	Sandy Clay Loam Soil Spiked with IPD072Aa Protein	29[a]	58.6	12	0.158 ± 0.0900	0.1 - 0.4
4	Control Sandy Clay Loam Soil	28[a]	3.57	27	0.389 ± 0.167	0.1 - 0.9
5	Silt Loam Soil Spiked with IPD072Aa Protein	28[a]	50.0	14	0.143 ± 0.0852	0.1 - 0.4
6	Control Silt Loam Soil	29[a]	0	29	0.448 ± 0.124	0.2 - 0.7

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% pooled soil incorporated into dry carrier (by carrier dry weight). Treatments 2, 4, and 6 contained soil spiked with ultrapure water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

Table 257. Summary of *Diabrotica virgifera virgifera* Results (Initiated with Day 7 Soil)

Treatment	Treatment Description	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
					Mean ± Standard Deviation	Range
1	Loam Soil Spiked with IPD072Aa Protein	30	0	30	0.223 ± 0.125	0.1 - 0.5
2	Control Loam Soil	30	0	30	0.333 ± 0.132	0.1 - 0.6
3	Sandy Clay Loam Soil Spiked with IPD072Aa Protein	29[a]	0	29	0.352 ± 0.143	0.1 - 0.7
4	Control Sandy Clay Loam Soil	30	0	30	0.343 ± 0.125	0.1 - 0.6
5	Silt Loam Soil Spiked with IPD072Aa Protein	30	6.67	28	0.325 ± 0.140	0.1 - 0.7
6	Control Silt Loam Soil	30	0	30	0.373 ± 0.184	0.1 - 0.7

Note: Treatments 1-6 for the *Diabrotica virgifera virgifera* bioassays contained approximately 20% pooled soil incorporated into dry carrier (by carrier dry weight). Treatments 2, 4, and 6 contained soil spiked with ultrapure water.

[a] Organisms counted as missing during the bioassay were not included in the total number of observations for a given treatment.

K.1. Analytical Phase

Western blot analysis was used to visually assess the dissipation of IPD072Aa protein in loam, sandy clay loam, and silt loam soils over time.

Sample Collection

On the day of soil spiking (Day 0) and after 1, 2, 3, 4, 7, and 14 days of incubation, three randomly selected samples of loam, sandy clay loam, and silt loam soil spiked with IPD072Aa protein were collected and extracted for western blot analysis.

Sample Extraction and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

On the day of collection, samples of IPD072Aa-spiked soils (~250 mg) were each extracted in 600 µl of 1X lithium dodecyl sulfate (LDS)/dithiothreitol (DTT) sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing dithiothreitol, and 65% water). In addition, on the day of soil spiking, sub-samples of the test substance were prepared for SDS-PAGE by combining 65% IPD072Aa protein test substance, 25% 4X LDS sample buffer, and 10% reducing agent. All samples were heated at 90-100 °C for 5 minutes and stored frozen (-80 °C freezer unit).

Prior to SDS-PAGE, samples (two per timepoint for each IPD072Aa-spiked soil type) were thawed and diluted, after being heated at 90-100 °C for 3 minutes. Samples were then loaded into 4-12% Bis-Tris gels along with pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) to provide a visual verification that migration was within the expected range of the predicted molecular weight (~10 kDa). Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) until the dye front was near the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gels were each assembled into a nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gels to nitrocellulose membranes for 7 minutes with a pre-set program (P3).

Following protein transfer, the membranes were blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 1 hour at ambient temperature. Before the blocking step, the membranes were washed with PBST three times for 1 minute each to reduce the background. The blocked membranes were incubated

with an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:20,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. Following primary antibody incubation, the membranes were washed with PBST four times for 5 minutes each. The membranes were incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate; Promega Corporation) diluted 1:20,000 in PBST containing 1% w/v non-fat dry milk for 1 hour at ambient temperature. The membranes were then washed with PBST four times for 5 minutes each. Each blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system. The IPD072Aa protein (monomer) migrates at a molecular weight of approximately 10 kDa.

K.2. Bioassay Details

Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 24-well bioassay plate and contained 3 replicates from each treatment. Each treatment was fed to a target of 30 *WCR* individuals. Bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 4 of the bioassay with soil aliquots that had been incubated an additional 4 days after the initiation of the bioassay. The first bioassay used soils from Day 0 and Day 4 after soil spiking. The second bioassay used soils from Day 7 and Day 11 after soil spiking. Bioassays were complete after 7 days of feeding. Mortality was assessed and surviving organisms were individually weighed. Mortality results were used to demonstrate the loss of insecticidal activity for the IPD072Aa protein in each soil type.

The bioassay acceptability criterion indicated the bioassay may be terminated and repeated if:

The combined number of dead and missing organisms exceeds 30% for the bioassay controls containing water-spiked soils.

Diet Treatment Preparation

To begin each bioassay and 4 days after each bioassay was initiated, samples from each soil type were collected and used to create artificial diet to feed *WCR*. On each day of diet preparation, three randomly selected aliquots of the IPD072Aa protein-spiked soil and three control soil samples for each soil type were removed from the environmental chamber, pooled by treatment, and mixed with carrier at approximately 20% soil by dry weight of carrier. Ultrapure (American Society for Testing and Materials ASTM Type 1) water was added to the dry soil-carrier mix at a 2.51:1 ratio (*i.e.*, 2.51 ml water to 1 g carrier), generating Treatments 1-6.

WCR Bioassays

WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was recorded by study personnel. A separate batch of *WCR* eggs for each bioassay was incubated in an environmental chamber until the eggs hatched. *WCR* neonates were used in the bioassays within 24 hours of hatching.

On Day 0 of each respective bioassay, approximately 300 µl (*i.e.*, 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of 24-well bioassay plates. One *WCR* neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film, and two small holes were poked over each well to allow for ventilation. Bioassays were conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4 of each respective bioassay, new plates were prepared with fresh diet as described for Day 0, living *WCR* larvae were transferred to the new plates, missing or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed. Only wells that contained one organism were included in the total number of observed individuals; organisms recorded as missing from a well were excluded from statistical analysis.

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PIONEER®

Petition for the Determination of Nonregulated Status for Insect Resistant and Herbicide-Tolerant DP23211 Maize

Supplement 1: Characterization and Equivalency of the IPD072Aa Protein in DP23211 Maize

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OECD Unique Identifier: DP-Ø23211-2

No Confidential Business Information is Contained in this Document

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article, DP-23211-2 maize (DP23211) not be regulated under 7 CFR §340.

Sally A. Catron 9/11/20

Sally A. Catron Date

Release of Information

Pioneer Hi-Bred International, Inc. ("Pioneer", a member of the Corteva Agriscience Group of Companies), is submitting the information in this assessment for review by the USDA as part of the regulatory process. By submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C. section 522 or USDA's implementation regulations (7 CFR §340). In the event that USDA receives a FOIA request covering all or some of the information in this submission, Pioneer expects that, in advance of the release of the document(s), USDA will provide Pioneer with a copy of the material proposed to be released and the opportunity to object to the release of any information based upon appropriate legal grounds, e.g., responsiveness, confidentiality, or otherwise. Pioneer expects that no information that has been identified as confidential business information (CBI) will be provided to any third party. Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without prior notice and consent from Pioneer.

Certification

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article, DP-Ø23211-2 maize (DP23211) not be regulated under 7 CFR §340.

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners, which are favorable and unfavorable to the petition.

9/11/20

Date

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A. Characterization of Microbially Derived IPD072Aa Protein for use in Non-Target and Spectrum Organism Studies (PCF-0040)

The objective of this study was to characterize the IPD072Aa protein derived from a microbial expression system in support of safety assessment studies.

Protein lot # PCF-0040 was used in the following studies presented in the DP23211 Maize petition:

Heat stability, soil dissipation, Bobwhite Quail, Collembola, Honeybee (Adult and Larval), Superworm, Parasitic Hymenoptera, Lacewing, Dose Response Western Corn Rootworm, Southern Corn Rootworm, Superworm, and Synergism.

Characterization of the IPD072Aa protein was achieved using sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] analysis, western blot analysis, protein glycosylation analysis, amino acid composition analysis, mass determination of the intact protein by mass spectrometry, mass determination of chymotryptic peptides by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS/MS), N-terminal amino acid sequencing, and bioactivity assay methods. The endotoxin content of the IPD072Aa protein was also determined. Results were as follows:

SDS-PAGE analysis demonstrated the microbially derived IPD072Aa protein migrated as a predominant band consistent with the expected molecular weight of approximately 10 kilodaltons (kDa). Densitometry analysis indicated that the purity of the microbially derived IPD072Aa protein was a single band on the gel and purity is therefore reported as > 95% on a total protein basis.

Western blot analysis demonstrated the expected immunoreactivity for the microbially derived IPD072Aa protein.

Protein glycosylation was not detected for the microbially derived IPD072Aa protein using a glycoprotein staining assay.

Amino acid composition analysis determined that the concentration of the microbially derived IPD072Aa protein was 0.80 milligrams (mg) of protein per mg of lyophilized powder.

The predominant mass obtained by MALDI-MS analysis of the protein was 9549.3 daltons (Da), consistent with the expected mass of 9548.9 Da for the microbially derived IPD072Aa protein based on the protein sequence.

The matched peptides identified with MALDI- MS analysis of the chymotrypsin-digested IPD072Aa protein account for 100% of the expected IPD072Aa protein amino acid sequence.

N-terminal amino acid sequence analysis demonstrated that the primary sequence (HMGITVTNNS) was consistent with amino acid residues 1-10 of the expected theoretical sequence of the microbially derived IPD072Aa protein.

The bioactivity analysis demonstrated that the microbially derived IPD072Aa protein had insecticidal activity toward a target insect, western corn rootworm (*Diabrotica virgifera virgifera*).

An endotoxin assay demonstrated that the endotoxin content in the microbially derived IPD072Aa protein was less than 250 endotoxin units per mg of protein.

The microbially derived IPD072Aa protein had the expected molecular weight, immunoreactivity, amino acid sequence, bioactivity, and lack of glycosylation and demonstrated acceptable endotoxin content. The protein lot analyzed in this study is hereby considered characterized for use in regulatory studies.

Materials

Test Substance

The IPD072Aa protein was produced at Pioneer Hi-Bred International, Inc. The protein was expressed in an *E. coli* protein expression system as a fusion protein with an N-terminal His tag. The tagged protein was purified using Ni-NTA affinity chromatography. The fusion tag was cleaved by immobilized trypsin and then removed by Ni-NTA affinity chromatography. Following purification and His tag removal, tangential flow filtration was used to change the buffer to 50 mM ammonium bicarbonate. After lyophilization and mixing, a lot number (PCF-0040) was assigned.

The test substance consisted of purified IPD072Aa protein in lyophilized powder.

Bioassay and Positive Control Solutions

The bioassay control solution used in the sensitive insect bioassay consisted of ultrapure water (American Society for Testing and Materials [ASTM] Type 1 water, referred to as water).

The positive control solution used in the sensitive insect bioassay consisted of boric acid in water.

Carrier

The carrier into which the dosing solutions were incorporated was a dry artificial insect diet prepared by Pioneer for the WCR bioassay.

Test System

The test system for the bioassay activity portion of the study was neonate western corn rootworm (WCR; *Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Methods

SDS-PAGE Analysis

A 5.0-mg lyophilized IPD072Aa protein sample was solubilized in 4.0 ml of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water) and heated at 90-100 °C for 5 minutes prior to SDS-PAGE analysis.

The prepared protein samples were analyzed using 4-12% Bis-Tris gels. For Coomassie staining and glycosylation staining, 1 µg of IPD072Aa protein was loaded. For western blot analysis, 5 ng of IPD072Aa protein was loaded. For mass spectrometry analyses, 4 µg of IPD072Aa protein was loaded. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight. Electrophoresis was conducted using a Mini-Cell Electrophoresis System with 1X MES SDS running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for mass spectrometry of chymotryptic peptides.

For Coomassie staining, the gel was washed with water three times for a minimum of 5 minutes each, and stained with GelCode Blue Stain Reagent for 62-69 minutes. Following staining, the gel was de-stained with water four times for a minimum of 5 minutes each or until the gel background was clear. Proteins were detected as blue-colored bands on the gels. The gel image was captured electronically using an imaging system. Densitometry analysis of the gel was conducted to evaluate the purity of the IPD072Aa protein based on the relative intensity of the IPD072Aa protein band within the lane using molecular imaging software.

Western Blot Analysis

Following SDS-PAGE, the resulting gel was assembled into a mini nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gel to the nitrocellulose membrane for 7 minutes with a pre-set program (P3).

Following protein transfer, the membrane was blocked in phosphate buffered saline with polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 65 minutes at ambient temperature. Before and after the blocking step, the membranes were washed with PBST three times for 5 minutes each to reduce the background. The blocked membrane was incubated in a IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:10,000 in PBST containing 1% (w/v) non-fat dry milk for 60 minutes at ambient temperature. Following primary antibody incubation, the membrane was washed with PBST three times for 5 minutes each. The membrane was incubated in a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient temperature. The membrane was then washed with PBST three times for 5 minutes each. The blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

Protein Glycosylation Analysis

The Pierce Glycoprotein Staining Kit was used to determine whether the IPD072Aa protein was glycosylated. The IPD072Aa protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor), were run by SDS-PAGE as described in Methods Section A.

Following electrophoresis, the gel was washed with water twice for 5 minutes each wash, fixed with 50% methanol for 30-35 minutes, and washed twice with 3% acetic acid for 10-15 minutes each wash. The gel was then incubated with oxidizing solution for 15-20 minutes and washed three times with 3% acetic acid for 5-7 minutes each wash. The gel was incubated with glycoprotein staining reagent for 15-20 minutes and then incubated in a reducing reagent for 5-7 minutes. The gel was then washed two times with 3% acetic acid for 5-7 minutes each wash and then rinsed in water once for 5 minutes. Glycoproteins were detected as magenta colored bands on the gel.

Following glycoprotein detection, the image of the gel was captured electronically. The same gel was then stained with GelCode Blue stain reagent for 60 minutes followed by three washes with water (minimum 5 minutes each wash) to visualize all protein bands. The image of the GelCode stained gel was then captured electronically.

Amino Acid Composition Analysis

Three individually weighed samples of the IPD072Aa lyophilized protein were solubilized in water and shipped to Alphalyse for amino acid composition analysis.

Each sample was analyzed using acid hydrolysis conducted for 20 hours at 110 °C in 6 M HCl, 0.1% phenol, and 0.1% thioglycolic acid. The hydrolysis took place under reduced pressure in an atmosphere of argon. Identification and quantification of the amino acids was conducted on a BioChrom 30 amino acid analyzer using ion exchange chromatography, post-column derivatization with ninhydrin, and detection at two wavelengths, 570 nm and 440 nm. A known amount of sarcosine was added as an internal control standard. The common 20 amino acids were determined, with the exception of tryptophan, which degrades under the conditions of analysis, and cysteine, for which yield is variable. Asparagine was determined as aspartic acid, and glutamine was determined as glutamic acid.

Determination of Molecular Mass by Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) Analysis

A sample (5.1 mg) of the IPD072Aa protein was solubilized in 4.1 ml of a solution of 0.5 mM ammonium acetate and 3% methanol (Alphalyse ESI MS Solution), and sent to Alphalyse for determination of intact molecular weight by MALDI-MS analysis. The IPD072Aa protein sample was further purified using Millipore C4 ZipTips, then mixed with 2,5-dihydroxyacetonphonone/diammonium hydrogen citrate matrix, spotted onto a Bruker Big Anchor target and analyzed with an Autoflex Speed MALDI TOF/TOF mass spectrometer in linear mode using Compas 1.4 control and processing software. The mass spectra were calibrated by external quadratic calibration using Bruker Protein Standard 1. The sample mass is calculated from the least charged ion within the calibrated range of the mass spectrum.

Protein Identification by MALDI-MS/MS Peptide Sequencing

A 5.0-mg lyophilized IPD072Aa protein sample was solubilized in 2.0 ml of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water) and heated at 90-100 °C for 5 minutes prior to SDS-PAGE analysis. Following SDS-PAGE, Coomassie staining, and imaging of the gel using the methods as described in Methods Section A, the IPD072Aa protein bands were excised from the gel and sent to Alphalyse for peptide sequencing. The protein samples were reduced and alkylated with iodoacetamide (*i.e.*, carbamidomethylated) and subsequently digested with trypsin and chymotrypsin. The resulting peptides were spotted directly onto an anchorchip target or were concentrated on a C18 ZipTip micropurification column and eluted onto an anchorchip target for analysis on a Bruker Autoflex Speed MALDI TOF/TOF instrument. The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination.

N-Terminal Amino Acid Sequence Analysis

A sample (5.1 mg) of the IPD072Aa protein was solubilized in 4.1 ml of a solution of 0.5 mM ammonium acetate and 3% methanol (Alphalyse ESI MS Solution), and sent to Alphalyse for Edman N-terminal amino acid sequencing using an ABI Procise 494 sequencer (Applied Biosystems, Inc.) equipped with an online high-performance liquid chromatography system.

Bioactivity Assay

The biological activity of the IPD072Aa protein was evaluated by conducting a 7-day bioassay using *Diabrotica virgifera virgifera*. The *D. virgifera virgifera* bioassay utilized a randomized complete block design containing four blocks. Each block consisted of a 24-well tissue culture plate containing eight wells of each artificial insect diet. The bioassay included a total of 32 replicates per treatment (8 replicates per block x 4 blocks = 32 replicates). *D. virgifera virgifera* neonates were exposed to the following three artificial insect diets: the bioassay control diet containing a dosing solution of water, the test diet containing a targeted concentration of 100 ng IPD072Aa protein/mg diet wet weight, and the positive control diet, containing a targeted concentration of 4285 ng boric acid/mg diet wet weight.

D. virgifera virgifera neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, a 10.1-mg sample of lyophilized IPD072Aa was solubilized in 4.04 ml of chilled water resulting in an IPD072Aa concentration of 2.0 mg/ml. The test dosing solution was then prepared at a concentration of 140 µg IPD072Aa protein/ml. The bioassay and positive control dosing solutions were also prepared and each dosing solution was combined with carrier at a ratio of approximately 2.51 parts solution to one part dry carrier. Approximately 300 µl of freshly prepared diets were dispensed into individual wells of the tissue culture plates and a *D. virgifera virgifera* neonate was placed in each well. After infestation, each plate was sealed with heat-sealing film and two small holes were punched over each well to allow for ventilation. Infested plates were placed in a dark environmental chamber at a setting of 21 °C and 65% relative humidity. Due to a calculation error, the actual concentration of the positive control diet was discovered to be 1176 ng boric acid/mg diet wet weight and fed in the bioassay for Days 0-3. On Day 4, new tissue culture plates were prepared using the same procedures used on Day 0, with the exception that the positive control diet contained 4285 ng boric acid/mg diet wet weight. Living *D. virgifera virgifera* larvae were transferred to the new plates and the plates were

returned to the environmental chamber. At the end of the 7-day feeding period, individual larvae were assessed for mortality and surviving larvae were removed from the bioassay wells and individually weighed.

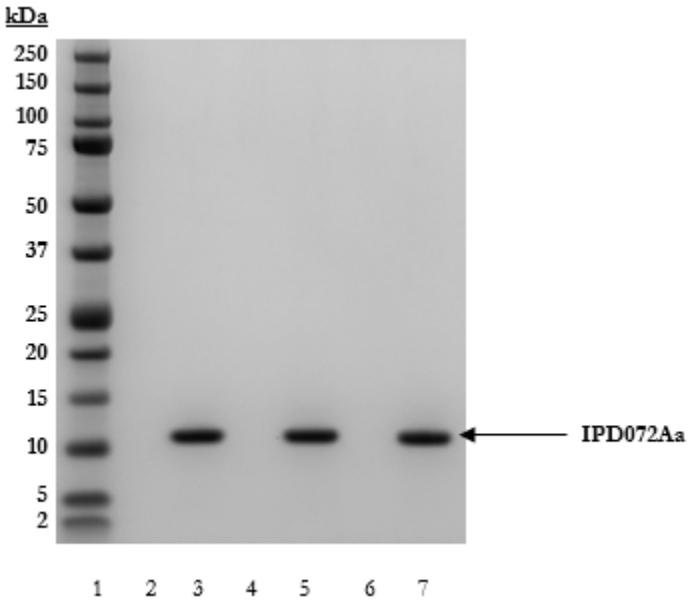
Determination of Endotoxin Content

A 5.2-mg sample of the lyophilized IPD072Aa protein powder was solubilized in 2.08 ml of endotoxin free water, resulting in a IPD072Aa protein concentration of 2.0 mg/ml. Endotoxin content was determined using a gel blot assay for the detection of Gram negative bacterial endotoxins with the Limulus Amebocyte Lysate Endosafe diagnostic kit (Charles River Laboratories, Inc., Charleston, SC, USA). Samples were diluted with endotoxin-free water to 1:100, 1:250, 1:500, 1:1000, 1:2000, 1:5000, and 1:10,000. A 200 µl sample of each dilution was distributed to an endotoxin test vial which was then incubated in a 37 °C water bath for 60 (±1) minutes. The endotoxin content in the protein was determined and calculated from the endotoxin concentration in the sample with the lowest dilution that did not cause coagulation.

Results and Discussion

SDS-PAGE Analysis

SDS-PAGE analysis demonstrated the IPD072Aa protein migrated as a predominant band consistent with the expected molecular weight of approximately 10 kDa (Figure 1). Densitometry analysis indicated that the purity of the IPD072Aa protein was a single band and is therefore reported as > 95% on a total protein basis.



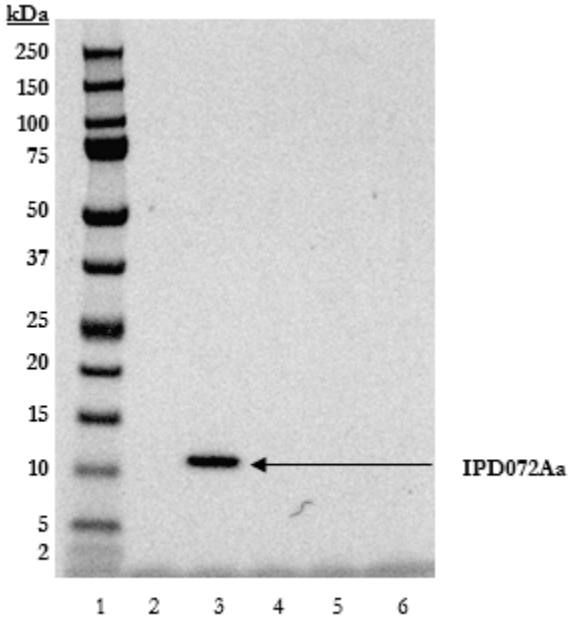
Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS Sample Buffer Blank
3	Microbially Derived IPD072Aa Protein (1 µg)
4	1X LDS Sample Buffer Blank
5	Microbially Derived IPD072Aa Protein (1 µg)
6	1X LDS Sample Buffer Blank
7	Microbially Derived IPD072Aa Protein (1 µg)

[a] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 1. SDS-PAGE Analysis of the Microbially Derived IPD072Aa Protein

Western Blot Analysis

Western blot analysis demonstrated that the IPD072Aa protein was immunoreactive to a IPD072Aa polyclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 10 kDa (Figure 2).



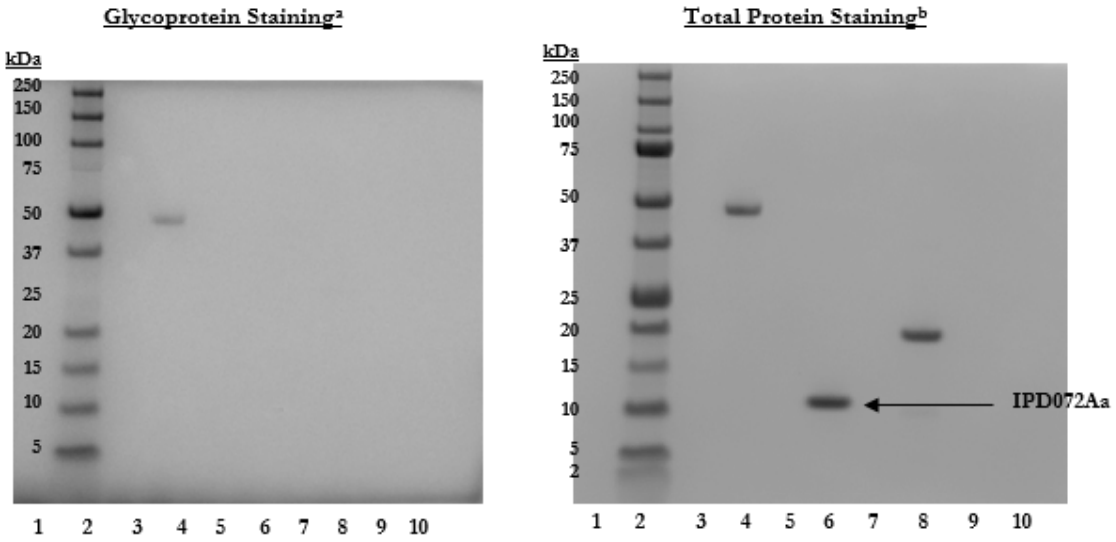
Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS Sample Buffer Blank
3	Microbially Derived IPD072Aa Protein (5 ng)
4	1X LDS Sample Buffer Blank
5	1X LDS Sample Buffer Blank
6	1X LDS Sample Buffer Blank

[a] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 2. Western Blot Analysis of the Microbially Derived IPD072Aa Protein

Protein Glycosylation Analysis

Glycosylation was not detected for the IPD072Aa protein. The horseradish peroxidase positive control was stained and clearly visible as a magenta-colored band. The soybean trypsin inhibitor negative control was not stained (Figure 3).



Lane	Sample Identification
1	1X LDS Sample Buffer Blank
2	Pre-stained Protein Molecular Weight Marker[c]
3	1X LDS Sample Buffer Blank
4	Horseradish Peroxidase (1 µg)
5	1X LDS Sample Buffer Blank
6	Microbially Derived IPD072Aa Protein (1 µg)
7	1X LDS Sample Buffer Blank
8	Soybean Trypsin Inhibitor (1 µg)
9	1X LDS Sample Buffer Blank
10	1X LDS Sample Buffer Blank

[a] Gel was stained with glycoprotein staining reagent.

[b] Gel was stained with glycoprotein staining reagent followed by staining with Coomassie Blue Reagent for total proteins.

[c] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 3. Glycosylation Analysis of the Microbially Derived IPD072Aa Protein

Amino Acid Composition

Amino acid composition analysis determined that the concentration of the IPD072Aa protein was 0.80 mg protein per mg lyophilized powder.

MALDI Mass Spectrometry Analysis

MALDI-MS analysis of the IPD072Aa protein obtained a mass of 9549.3 Da, consistent with the expected mass of 9548.9 Da based on the protein sequence.

MALDI- MS/MS Identification of Chymotryptic Peptides

The matched peptides identified with MALDI- MS/MS analysis of the chymotrypsin-digested IPD072Aa protein account for 100% of the expected IPD072Aa protein amino acid sequence (Figure 4, Table 1).

HMGITVTNNSSNPIEVAINHWGSDGDTSFFSVGNGKQETWDRSDSRGFVLSLKKNGAQHPY YVQASSKIEVDNNAVKDQGRLIEPLS

Gray shading	Gray-shaded type indicates microbially derived IPD072Aa peptides identified using MALDI MS/MS analysis.
Amino acid residue abbreviations	alanine (A), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Figure 4. Amino Acid Sequence of the Microbially Derived IPD072Aa Protein Indicating Chymotryptic Peptides Identified Using MALDI- MS/MS Analysis

Table 1. Identified Chymotryptic Peptides of the Microbially Derived IPD072Aa Protein Using MALDI-MS Analysis

IPD072Aa Amino Acid Residue Position	IPD072Aa Calculated Peptide Mass [M][a]	Observed IPD072Aa Peptide Mass [M+H] (input)[a]	Identified Peptide Sequence	Modifications (If Applicable)
1- 30	3246.47	3247.53	HMGITVTNNSSNPIEVAINHWGSDGDTSFF	None
1- 30	3262.46	3263.52	HMGITVTNNSSNPIEVAINHWGSDGDTSFF	Oxidation (M)
31 – 40	1104.52	1105.55	SVGNGKQETW	None
31 – 48	2024.93	2025.98	SVGNGKQETWDRSDSRGF	None
31 – 50	2237.08	2238.13	SVGNGKQETWDRSDSRGFVL	None
41 – 48	938.42	939.45	DRSDSRGF	None
41 – 50	1150.57	1151.61	DRSDSRGFVL	None
49 – 61	1453.80	1454.84	VLSLKKNGAQHPY	None
51 – 61	1241.65	1242.69	SLKKNGAQHPY	None
53 - 61	1041.54	1042.57	KKNGAQHPY	None
62 - 87	2872.49	2873.57	YVQASSKIEVDNNAVKDQGRLIEPLS	None

[a] The mass [M] values can be converted to "[M+H]" by addition of 1.00 to account for protonation.

N-Terminal Amino Acid Sequence Analysis Table 2

N-terminal amino acid sequence analysis of the IPD072Aa protein obtained a primary sequence (HMGITVTNNS) matching amino acid residues 1-10 of the expected protein sequence.

Table 2. N-Terminal Amino Acid Sequence of the Microbially Derived IPD072Aa Protein

Theoretical IPD072Aa Sequence	H – M – G – I – T – V – T – N – N - S
Detected Primary Sequence	H – M – G – I – T – V – T – N – N - S

Bioactivity Assay

Bioactivity analysis demonstrated that the microbially derived IPD072Aa protein had insecticidal activity toward a target insect, *D. virgifera virgifera*. Summary of *D. virgifera virgifera* mortality data consisted of the calculation of dead larvae divided by the total number of observed larvae at the end of the study and multiplied by 100. Mortality was 97% in *D. virgifera virgifera* fed a diet containing IPD072Aa protein compared to 13% in *D. virgifera virgifera* fed the bioassay control diet.

Table 3. Summary Analysis of Western Corn Rootworm Larval Mortality Results

Treatment ID	Treatment Description	Total number of observed larvae	Mortality (%)	Average Weight (mg)	Standard Deviation (mg)
1	Bioassay Control diet	32	13.0%	0.5	0.1
2	100 ng IPD072Aa/mg diet[1]	32	97.0%	0.1	0.1
3	4285 ng Boric Acid/mg diet[1]	32	63.0%	0.2	0.1
[1]Concentrations calculated based on wet weight of diet					

Endotoxin Assay

The endotoxin content in the IPD072Aa protein is less than 250 endotoxin units per mg protein.

Conclusion

The microbially derived IPD072Aa protein had the expected molecular weight, immunoreactivity, amino acid sequence, bioactivity, and lack of glycosylation and demonstrated acceptable endotoxin content. The protein lot analyzed in this study is hereby considered characterized for use in regulatory studies.

B. Characterization of Microbially Derived IPD072Aa Protein for use in Non-Target and Spectrum Organism Studies (PCF-0037)

The objective of this study was to characterize IPD072Aa protein derived from a microbial expression system.

Protein lot # PCF-0040 was used in the following insect bioactivity studies presented in the DP23211 Maize petition:

Spectrum of activity insects: European Corn Borer, Corn Ear Worm, Codling Moth, Painted Lady, Colorado Potato Beetle, Convergent Lady Beetle, Mealworm, Mealybug Destroyer, Mexican Bean Beetle, Pink Spotted Lady Beetle, Red Flour Beetle, and Rove Beetle.

Characterization of the IPD072Aa protein was achieved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, western blot analysis, protein glycosylation analysis, amino acid composition analysis, mass determination of the intact protein by mass spectrometry, mass determination of chymotryptic peptides by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), and N-terminal amino acid sequencing. The bioactivity and endotoxin content of the IPD072Aa protein was also determined. Results were as follows:

SDS-PAGE analysis demonstrated the microbially derived IPD072Aa protein migrated as a predominant band consistent with the expected molecular weight of approximately 10 kilodaltons (kDa). Densitometry analysis indicated that the purity of the microbially derived IPD072Aa protein was a single band on the gel and purity is therefore reported as > 95% on a total protein basis.

Western blot analysis demonstrated the expected immunoreactivity for the microbially derived IPD072Aa protein.

Protein glycosylation was not detected for the microbially derived IPD072Aa protein using a glycoprotein staining assay.

Amino acid composition analysis determined that the concentration of the microbially derived IPD072Aa protein was 0.82 mg of protein per mg of lyophilized powder.

Molecular mass determination by MALDI mass spectrometry analysis of the protein obtained a predominant mass of 9549.8 daltons (Da), consistent with the expected mass of 9548.9 Da for the microbially derived IPD072Aa protein based on the protein sequence.

The peptides identified with peptide mapping by mass spectrometry analysis of the chymotrypsin-digested IPD072Aa protein accounted for 100% of the expected IPD072Aa amino acid sequence.

N-terminal amino acid sequence analysis demonstrated that the primary sequence (HMGITVTNNS) was consistent with amino acid residues 1-10 of the expected theoretical sequence of the microbially derived IPD072Aa protein.

The bioactivity analysis demonstrated that the microbially derived IPD072Aa protein had insecticidal activity toward a target insect, western corn rootworm (*Diabrotica virgifera virgifera*).

An endotoxin assay demonstrated that the endotoxin content in the microbially derived IPD072Aa protein was less than 62.5 endotoxin units per mg of protein.

The microbially derived IPD072Aa protein had the expected molecular weight, immunoreactivity, amino acid sequence, bioactivity, and lack of glycosylation. Endotoxin content was also determined. The protein lot analyzed in this study is hereby considered characterized for use in regulatory studies.

Materials

Test Substance
The IPD072Aa protein was produced at Pioneer Hi-Bred International, Inc. The protein was expressed in an *E. coli* protein expression system as a fusion protein with an N-terminal His tag. The tagged protein was purified using Ni-NTA affinity chromatography. The fusion tag was cleaved by immobilized trypsin and then removed by Ni-NTA affinity chromatography. Following purification and His tag removal, tangential flow filtration was used to change the buffer to 50 mM ammonium bicarbonate. After lyophilization and mixing, a lot number (PCF-0037) was assigned to the protein. The IPD072Aa protein (PCF-0037) was further purified by anion exchange chromatography following resuspension of the lyophilized powder in 25 mM Tris pH 8.0. The buffer of the protein was then exchanged into 50 mM ammonium bicarbonate. The protein in solution was then lyophilized and the lot number (PCF-0037-AP) was assigned.

Bioassay and Positive Control Solutions
The bioassay control solution used in the sensitive insect bioassay consisted of ultrapure water (American Society for Testing and Materials [ASTM] Type 1 water, referred to as water).

The positive control solution used in the sensitive insect bioassay consisted of boric acid in water.

Carrier
The carrier into which the dosing solutions were incorporated was a dry artificial insect diet prepared by Pioneer for the WCR bioassay.

Test System
The test system for the bioassay activity portion of the study was neonate western corn rootworm (WCR; *Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae). The test system was chosen because WCR is an insect sensitive to IPD072Aa protein. WCR eggs were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) and identity was confirmed by study personnel.

Methods

SDS-PAGE Analysis

A 5.1-mg lyophilized IPD072Aa protein sample was solubilized in 2.09 ml of 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water) and heated at 90-100 °C for 5 minutes prior to SDS-PAGE analysis.

The prepared protein samples were analyzed using 4-12% Bis-Tris gels. For Coomassie staining and glycosylation staining, 1 µg of IPD072Aa protein was loaded. For western blot analysis, 5 ng of IPD072Aa protein was loaded. For mass spectrometry analyses, 4 µg of IPD072Aa protein was loaded. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight. Electrophoresis was conducted using a Mini-Cell Electrophoresis System with 1X MES running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for mass spectrometry of chymotryptic peptides.

For Coomassie staining, the gel was washed with water three times for a minimum of 5 minutes each, and stained with GelCode Blue Stain Reagent for 60-61 minutes. Following staining, the gel was de-stained with water four times for a minimum of 5 minutes each or until the gel background was clear. Proteins were detected as blue-colored bands on the gels. The gel image was captured electronically using an imaging system. Densitometry analysis of the gel was conducted to evaluate the purity of the IPD072Aa protein based on the relative intensity of the IPD072Aa protein band within the lane using molecular imaging software.

Western Blot Analysis

Following SDS-PAGE, the resulting gel was assembled into a mini nitrocellulose iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gel to the nitrocellulose membrane for 7 minutes with a pre-set program (P3).

Following protein transfer, the membrane was blocked in phosphate buffered saline containing polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 60 minutes at ambient temperature. Before and after the blocking step, the membrane was washed with PBST three times for 5 minutes each to reduce the background. The blocked membrane was incubated in an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted 1:5,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient temperature. Following primary antibody incubation, the membrane was washed with PBST four times for 5 minutes each. The membrane was incubated in a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient temperature. The membrane was then washed with PBST four times for 5 minutes each. The blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

Protein Glycosylation Analysis

A Pierce Glycoprotein Staining Kit was used to determine if the IPD072Aa protein was glycosylated. The IPD072Aa protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor) were run by SDS-PAGE as described in Methods Section A.

Following electrophoresis, the gel was washed with water twice for 5 minutes and fixed with 50% methanol for 30-35 minutes and washed twice with 3% acetic acid for 10-15 minutes each wash. The gel was then incubated with oxidizing solution for 15-20 minutes and washed three times with 3% acetic acid for 5-7 minutes each wash. The gel was incubated with glycoprotein staining reagent for 15-20 minutes and then incubated in a reducing reagent for 5-7 minutes. The gel was then washed three times with 3% acetic acid for 5 minutes each wash and then rinsed in water once for 5 minutes. Glycoproteins were detected as magenta-colored bands on the gel.

Following glycoprotein detection, the image of the gel was captured electronically. The same gel was then stained with GelCode Blue stain reagent for 63 minutes followed by three washes with water (minimum 5 minutes each wash) to visualize all protein bands. The image of the GelCode stained gel was then captured electronically.

Amino Acid Composition Analysis

Three individually weighed samples of the IPD072Aa lyophilized protein were solubilized in water and shipped to Alphalyse for amino acid composition analysis.

Each sample was analyzed using acid hydrolysis conducted for 20 hours at 110 °C in 6 M HCl, 0.1% phenol, and 0.1% thioglycolic acid. The hydrolysis took place under reduced pressure in an atmosphere of argon. Identification and quantification of the amino acids was conducted on a BioChrom 30 amino acid analyzer using ion exchange chromatography, post-column derivatization with ninhydrin, and detection at two wavelengths, 570 nm and 440 nm. A known amount of sarcosine was added as an internal control standard. The common 20 amino acids were determined, with the exception of tryptophan, which degrades under the conditions of analysis, and cysteine, for which yield is variable. Asparagine was determined as aspartic acid, and glutamine was determined as glutamic acid.

Molecular Mass Determination by MALDI Mass Spectrometry Analysis

A 5.0-mg sample of the IPD072Aa protein was solubilized in 4.1 ml of a solution of 0.5 mM ammonium acetate and 3% methanol, and sent to Alphalyse for determination of intact molecular weight by MALDI-MS analysis. The IPD072Aa protein sample was further purified using Millipore C18 Ziptips, then mixed with 2,5-dihydroxyacetonphonone/diammonium hydrogen citrate matrix and analyzed with an Autoflex Speed MALDI TOF/TOF mass spectrometer in linear mode using Compas 1.4 control and processing software. The mass spectra were calibrated by external quadratic calibration using Bruker Protein Standard 1. The sample mass is calculated from the least charged ion within the calibrated range of the mass spectrum.

Peptide Mapping by Mass Spectrometry Analysis

Following SDS-PAGE, Coomassie staining, and imaging of the gel using the methods as described in Methods Section A, the IPD072Aa protein band was excised from the gel and sent to Alphalyse for peptide sequencing. The protein sample was reduced and alkylated with iodoacetamide (*i.e.*, carbamidomethylated), and subsequently digested with chymotrypsin that cleaves after leucine, phenylalanine, tryptophan, and tyrosine residues. The resulting peptides were analyzed on a Bruker Autoflex Speed MALDI TOF/TOF instrument in positive reflector mode for accurate peptide mass determination. MALDI MS/MS was performed on some peptides for peptide fragmentation analysis, *i.e.*, partial sequencing. The MS and MS/MS spectra were combined and used for database searching using the Mascot software.

N-Terminal Amino Acid Sequence Analysis

A 5.0-mg sample of the IPD072Aa protein was solubilized in 4.1 ml of a solution of 0.5 mM ammonium acetate and 3% methanol, and sent to Alphalyse for Edman N-terminal amino acid sequencing using an ABI Procise 494 sequencer (Applied Biosystems, Inc.) equipped with an online high performance liquid chromatography system.

Bioactivity Analysis

The biological activity of the IPD072Aa protein was evaluated by conducting a 7-day bioassay using WCR, an insect sensitive to IPD072Aa protein. The WCR bioassay utilized a randomized complete block design containing four blocks. Each block consisted of a 24-well tissue culture plate containing eight wells of each artificial insect diet. The bioassay included a total of 32 replicates per treatment (8 replicates per block x 4 blocks = 32 replicates). WCR neonates were exposed to the following three artificial insect diets: the bioassay control diet containing a dosing solution of water, the test diet containing a targeted concentration of 100 ng IPD072Aa protein/mg diet wet weight, and the positive control diet, containing a targeted concentration of 4285 ng boric acid/mg diet wet weight.

WCR eggs were incubated in an environmental chamber until the eggs hatched. WCR neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, a 5.2-mg sample of lyophilized IPD072Aa was solubilized in 2.13 ml of chilled water resulting in an IPD072Aa concentration of 2.0 mg/ml. The test dosing solution was then prepared at a concentration of 140 µg IPD072Aa

protein/ml . The bioassay and positive control dosing solutions were also prepared and each dosing solution was combined with carrier at a ratio of approximately 2.5 parts solution to one part dry carrier. Approximately 300 µl of freshly prepared diets were dispensed into individual wells of the tissue culture plates and a WCR neonate was placed in each well. After infestation, each plate was sealed with heat-sealing film and two small holes were punched over each well to allow for ventilation. Infested plates were placed in a dark environmental chamber at a setting of 21 °C and 65% relative humidity. On Day 3 of the bioassay, new tissue culture plates were prepared using the same procedures used on Day 0, living WCR larvae were transferred to the new plates, and the plates were returned to the environmental chamber. Upon transfer and at the end of the 7-day feeding period, individual larvae were assessed for mortality. Surviving larvae were removed from the bioassay wells and individually weighed. Bioassay wells designated as containing diet but missing larvae at the time of mortality assessment were noted and those replicates were excluded from further analyses.

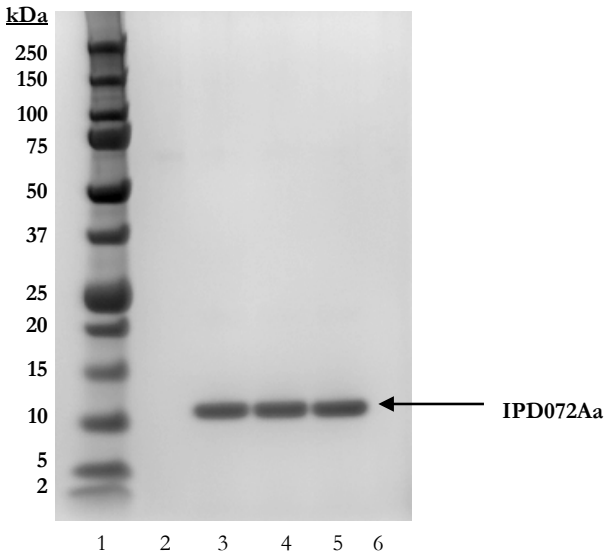
Determination of Endotoxin Content

A 5.0-mg sample of the lyophilized IPD072Aa protein was solubilized in 4.1 ml of endotoxin-free water, resulting in an IPD072Aa protein concentration of 1.0 mg/ml. Endotoxin content was determined using a gel clot assay for the detection of Gram-negative bacterial endotoxins with the Limulus Amebocyte Lysate Endosafe diagnostic kit. Samples were diluted with endotoxin-free water to 1:100, 1:250, 1:500, 1:1000, 1:2000, 1:5000, and 1:10,000. One 200-µl sample of each diluted sample was added to individual endotoxin test vials which were then incubated in a 37 °C water bath for 60 (±1) minutes. The endotoxin level was calculated from the results of the assay determined by the dilutions that coagulate.

Results and Discussion

SDS-PAGE Analysis Figure 1

SDS-PAGE analysis demonstrated the microbially derived IPD072Aa protein migrated as a predominant band consistent with the expected molecular weight of approximately 10 kilodaltons (kDa). Densitometry analysis indicated that the purity of the microbially derived IPD072Aa protein was a single band on the gel and purity is therefore reported as > 95% on a total protein basis (Figure 5).



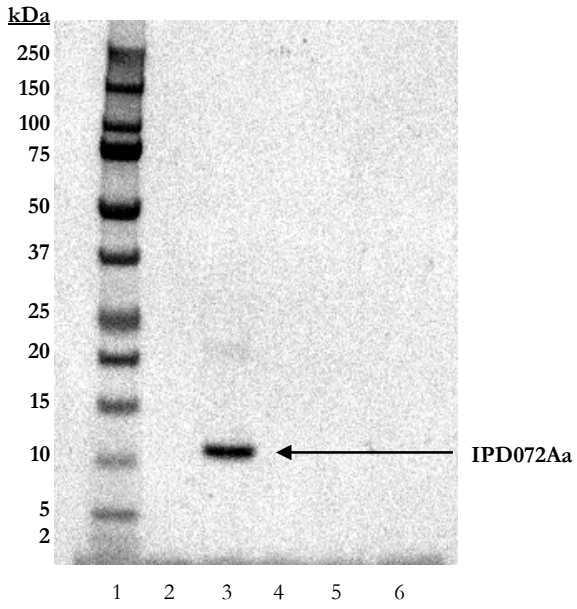
Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	1X LDS Sample Buffer Blank
3	Microbially Derived IPD072Aa Protein (1 µg)
4	Microbially Derived IPD072Aa Protein (1 µg)
5	Microbially Derived IPD072Aa Protein (1 µg)
6	1X LDS Sample Buffer Blank

Note: kilodalton (kDa) and microgram (µg). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 5. SDS-PAGE Analysis of the Microbially Derived IPD072Aa Protein

Western Blot Analysis

Western blot analysis demonstrated the expected immunoreactivity for the microbially derived IPD072Aa protein (Figure 6).



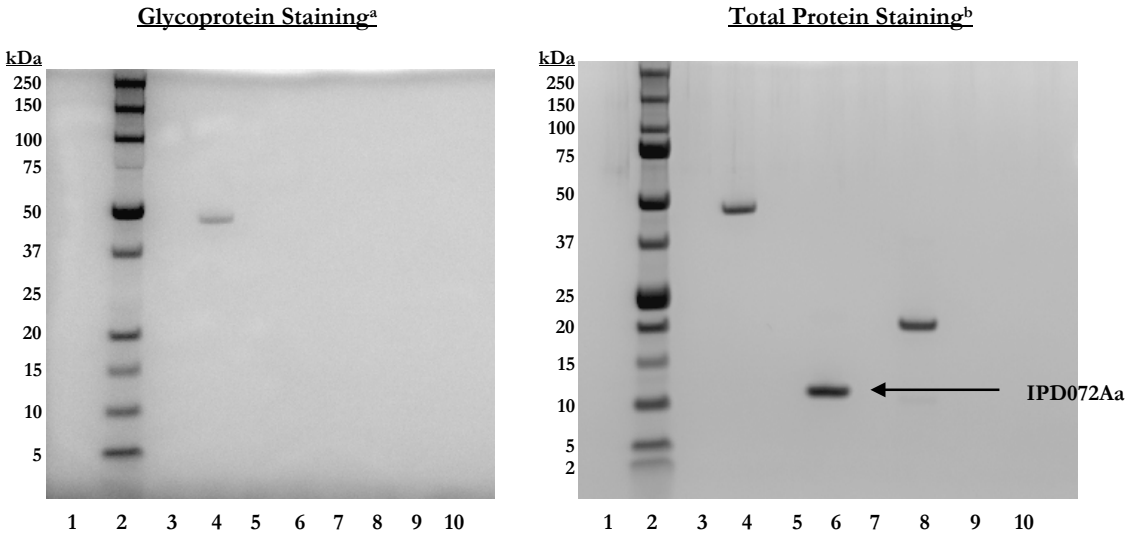
Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	1X LDS Sample Buffer Blank
3	Microbially Derived IPD072Aa Protein (5 ng)
4	1X LDS Sample Buffer Blank
5	1X LDS Sample Buffer Blank
6	1X LDS Sample Buffer Blank

Note: kilodalton (kDa) and nanogram (ng). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure 6. Western Blot Analysis of the Microbially Derived IPD072Aa Protein

A. Protein Glycosylation Analysis

Protein glycosylation was not detected for the microbially derived IPD072Aa protein using a glycoprotein staining assay (Figure 7).



Lane	Sample Identification
1	1X LDS Sample Buffer Blank
2	Pre-stained Protein Molecular Weight Marker
3	1X LDS Sample Buffer Blank
4	Positive Control Horseradish Peroxidase (1 µg)
5	1X LDS Sample Buffer Blank
6	Microbially Derived IPD072Aa Protein (1 µg)
7	1X LDS Sample Buffer Blank
8	Negative Control Soybean Trypsin Inhibitor (1 µg)
9	1X LDS Sample Buffer Blank
10	1X LDS Sample Buffer Blank

Note: kilodalton (kDa) and microgram (µg). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

[a] Gel was stained with glycoprotein staining reagent.

[b] Gel was stained with glycoprotein staining reagent followed by staining with Coomassie Blue Reagent for total proteins.

Figure 7. Glycosylation Analysis of the Microbially Derived IPD072Aa Protein

Amino Acid Composition Analysis

Amino acid composition analysis determined that the concentration of the microbially derived IPD072Aa protein was 0.82 mg of protein per mg of lyophilized powder.

Molecular Mass Determination by MALDI Mass Spectrometry Analysis

The predominant mass obtained by MALDI-MS analysis of the protein was 9549.8 daltons (Da), consistent with the expected mass of 9548.9 Da for the microbially derived IPD072Aa protein based on the protein sequence.

Peptide Mapping by Mass Spectrometry Analysis

The matched peptides identified with MALDI-MS analysis of the chymotrypsin-digested IPD072Aa protein account for 100% of the expected IPD072Aa amino acid sequence (Figure 8, Table 4).

HMGITVTNNSSNPIEVAINHWGSDGDTSFFSVGNGKQETWDRSDSRGFVLSLKKNGAQHPYYVQ
ASSKIEVDNNAVKDQGRLIEPLS

Gray shading	Gray-shaded type indicates microbially derived IPD072Aa peptides identified using MALDI MS/MS analysis.
Amino acid residue abbreviations	Alanine (A), arginine (R), asparagine (N), aspartic acid (D), glutamic acid (E), glutamine (Q), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Figure 8. Amino Acid Sequence of the Microbially Derived IPD072Aa Protein Indicating Chymotryptic Peptides Identified Using MALDI- MS Analysis

Table 4. Chymotryptic Peptides of the Microbially Derived IPD072Aa Protein Identified Using MALDI-MS Analysis

IPD072Aa Amino Acid Residue Position	IPD072Aa Theoretical Peptide Mass [M+H]	IPD072Aa Observed Peptide Mass [M+H] (input)	Identified Peptide Sequence
1- 30	3247.47	3247.33	HMGITVTNNSSNPIEVAINHWGSDGDTSFF
31 - 40	1105.52	1105.51	SVGNGKQETW
31 - 48	2025.93	2025.91	SVGNGKQETWDRSDSRGF
31 - 50	2238.08	2238.05	SVGNGKQETWDRSDSRGFVL
41 - 48	939.42	939.42	DRSDSRGF
41 - 50	1151.57	1151.56	DRSDSRGFVL
49 - 61	1454.80	1454.79	VLSLKKNGAQHPY
51 - 61	1242.65	1242.64	SLKKNGAQHPY
62 - 87	2873.49	2873.46	YVQASSKIEVDNNAVKDQGRLIEPLS

Note: alanine (A), arginine (R), asparagine (N), aspartic acid (D), glutamic acid (E), glutamine (Q), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

B. N-Terminal Amino Acid Sequence Analysis Table 2

N-terminal amino acid sequence analysis demonstrated that the primary sequence (HMGITVTNNS) was consistent with amino acid residues 1-10 of the expected theoretical sequence of the microbially derived IPD072Aa protein (Table 5).

Table 5. N-Terminal Amino Acid Sequence of the Microbially Derived IPD072Aa Protein

Theoretical IPD072Aa Sequence	H – M – G – I – T – V – T – N – N - S
Detected Sequence	H – M – G – I – T – V – T – N – N - S

Note: Asparagine (N), glycine (G), histidine (H), isoleucine (I), methionine (M), serine (S), threonine (T), and valine (V).

Bioactivity Analysis

Bioactivity analysis demonstrated that the microbially derived IPD072Aa protein had insecticidal activity toward a target insect, WCR. Summary of WCR mortality data consisted of the calculation of dead larvae divided by the total number of observed larvae at the end of the study and multiplied by 100. Mortality was 90.3% in WCR fed a diet containing IPD072Aa protein compared to 18.8% in WCR fed the bioassay control diet (Table 6).

Table 6. Summary Analysis of Western Corn Rootworm (*Diabrotica virgifera virgifera*) Larval Mortality Results

Treatment	Treatment Description	Total Number of Observed Larvae	Dead	Mortality (%)
1	Bioassay Control Diet	32	6	18.8
2	Diet Targeting 100 ng IPD072Aa Protein/mg	31[a]	28	90.3
3	Positive Control Diet	30[a]	30	100

Note: IPD072Aa protein concentration was calculated based on diet wet weight. Mortality is the final mortality at the end of the 7-day feeding period.

[a] Larvae counted as missing during the bioassay were not included in the total number of observed larvae for a given treatment.

Determination of Endotoxin Content

An endotoxin assay demonstrated that the endotoxin content in the microbially derived IPD072Aa protein was less than 62.5 endotoxin units per mg of protein.

CONCLUSION

The microbially derived IPD072Aa protein had the expected molecular weight, immunoreactivity, amino acid sequence, bioactivity, and lack of glycosylation. Endotoxin content was also determined. The protein lot analyzed in this study is hereby considered characterized for use in regulatory studies.