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Petition for the Determination of Nonregulated Status for Lepidopteran-Protected Maize MON 95379

The undersigned submits this petition under 7 CFR § 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR Part 340

July 21, 2020

(Revised January 24, 2024)

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Bayer Petition number: CR282-20U1

Submitted by:

DocuSigned by:
Heather Anderson
Signer Name: Heather Anderson
Signing Reason: I approve this document
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Heather M. Anderson, M.Sc.

Bayer U.S. – Crop Science
700 Chesterfield Pkwy West
Chesterfield, MO 63017
E-mail: heather.anderson@bayer.com

Prepared by:

P. Asiimwe, Ph.D., M. Bedair, Ph.D., J. Calcaterra, Ph.D., J. Fischer, Ph.D., G. Frierdich, B.S., D.A.B.T., I. Husic, Ph.D., W. Urquhart, Ph.D.

Contributors and/or Principal Investigators:

L. Barberis, B.S., E. Bretsnyder, M.S., C. Brown, Ph.D., G. Brown, B.S., B. Chen, Ph.D., E. Dickinson, B.S., T. Edrington, Ph.D., R. Dobert, Ph.D., J. Fridley, B.S., C. Jiang, Ph.D., S. Joshi, Ph.D., C. Kessenich, M.S., H. Kaur Bal Ph.D., D. Kendrick M.S., S. Levine, Ph.D., G. MacQuarrie, M.A., W. Li, Ph.D., S. Martinelli, Ph.D., M. Maven, M.S., G. Mueller, M.A., J. Nyangulu Ph.D., S. Reyna, M.S., K. Robinson, Ph.D., A. Silvanovich, Ph.D , K. Skottke, Ph.D., J. Tan, Ph.D., F. Zapata, M.S.

Contains no CBI.

RELEASE OF INFORMATION

Bayer is submitting the information in this petition for review by the USDA as part of the regulatory process. Bayer understands that the USDA complies with the provisions of the Freedom of Information Act (FOIA). In the event the USDA receives a FOIA request, pursuant to 5 U.S.C., § 552, and 7 CFR Part 1, covering all or some of the information in this petition, Bayer expects that, in advance of the release of the document(s), USDA will provide Bayer with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g., responsiveness, confidentiality, and/or competitive concerns. Bayer understands that a CBI-deleted copy of this information may be made available to the public in a reading room and upon individual request as part of a public comment period. Bayer also understands that when deemed complete, a copy of the petition may be posted to the USDA-APHIS BRS website or other U.S. government websites (e.g., www.regulations.gov). Except in accordance with the foregoing, Bayer does not authorize the release, publication or other distribution of this information without Bayer's prior notice and consent.

CERTIFICATION

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 petitioner that are unfavorable to the petition.

DocuSigned by:
Heather Anderson
Signer Name: Heather Anderson
Signing Reason: I approve this document
Signing Time: 25-Jan-2024 | 2:52:07 AM CET
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_____

Heather M. Anderson, M.Sc.
Senior Global Regulatory Manager

Address:
Bayer U.S. – Crop Science
700 Chesterfield Pkwy W,
Chesterfield, MO 63017

Tel: (636) 236-4499

EXECUTIVE SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S.

APHIS prior regulations codified in 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article. Newly promulgated 7 CFR § 340 regulations (issued May 18, 2020) state, in §340.1(c), that "All plants determined by APHIS to be deregulated pursuant to § 340.6 as that section was set forth prior to August 17, 2020 will retain their nonregulated status under these regulations." Thus, this petition seeks to gain a deregulation determination under those prior regulations and thus be exempt from the newly issued 7 CFR § 340 regulations.

Bayer is submitting this request to APHIS for a determination of nonregulated status for the new biotechnology-derived maize product, MON 95379, any progeny derived from crosses between MON 95379 and conventional maize, and any progeny derived from crosses of MON 95379 with biotechnology-derived maize that have previously been granted nonregulated status under 7 CFR Part 340 or determined in the future to not pose a plant pest risk via a regulatory status review or otherwise deemed to be exempt from 7 CFR Part 340.

Product Description

Insect-protected maize MON 95379 was developed to produce two insecticidal proteins, Cry1B.868 and Cry1Da_7, which protect against feeding damage caused by targeted lepidopteran insect pests. Cry1B.868 is a chimeric protein comprised of domains I and II from Cry1Be (*Bacillus thuringiensis, Bt*), domain III from Cry1Ca (*Bt* subsp. *aizawai*) and C-terminal protoxin domain from Cry1Ab (*Bt* subsp. *kurstaki*). Cry1Da_7 is a modified Cry1Da protein derived from *Bt* subsp. *aizawai*.

MON 95379 was developed to provide growers in South America an additional tool for controlling target lepidopteran maize pests, including fall armyworm resistant to current *Bt* technologies. MON 95379 will be combined through traditional breeding with other deregulated traits to provide protection against both above-ground and below-ground maize pests, as well as herbicide tolerance. These next generation combined-trait maize products will offer broader grower choice, improved production efficiency, increased pest control durability, and enhanced grower profit potential.

MON 95379 will not be commercialized in the U.S., but is intended to only be cultivated in small-scale breeding, testing, and seed increase nurseries to develop seed of products that will be sold in other countries, primarily in South America. These intended cultivation uses will be subject to the terms and conditions of an EPA seed increase registration, which we have proposed to be limited to no more than 100 acres per growing season across Nebraska, Hawaii, and Iowa. In the course of developing these products containing MON 95379, Bayer may also develop breeding stacks in

which MON 95379 will be combined with other traits for commercialization outside the United States. To support subsequent global regulatory submissions of these stack products, from time to time Bayer may conduct confined, small-scale field trials that will be planted in additional counties/states that are assessed under the standard USDA-BRS permitting process. Trials and trial material will be managed according to the conditions of applicable BRS permits and terms of an EPA seed increase registration, if applicable.

Data and Information Presented Confirms the Lack of Plant Pest Potential and the Food and Feed Safety of MON 95379 Compared to Conventional Maize

The data and information presented in this petition demonstrate MON 95379 is agronomically, phenotypically, and compositionally comparable to commercially cultivated maize, with the exception of the introduced trait. Moreover, the data and information presented demonstrate MON 95379 is not expected to pose a greater plant pest risk, including weediness, or adverse affects on non-target organisms beneficial to agriculture or maize agronomic practices compared to conventional maize. The food, feed, and environmental safety of MON 95379 was confirmed based on multiple, well-established lines of evidence:

- Maize is a familiar crop that does not possess any of the attributes commonly associated with weeds and has a history of safe consumption. The conventional control used for the transformation process was included in studies to serve as an appropriate basis of comparison for MON 95379.

- A detailed molecular characterization of the inserted DNA demonstrates a single, intact copy of the T-DNA insert in a single locus within the maize genome.

- Extensive evaluation of the Cry1B.868 and Cry1Da_7 proteins expressed in MON 95379 confirms they are unlikely to be human and other mammalian toxins or allergens.

- A compositional assessment supports the conclusion that MON 95379 grain and forage is compositionally equivalent to grain and forage of conventional maize.

- An extensive evaluation of MON 95379 phenotypic and agronomic characteristics and environmental interactions demonstrates MON 95379 has no increased plant pest risk potential compared to conventional maize.

- A non-target organism (NTO) assessment supports the conclusion that Cry1B.868 and Cry1Da_7 proteins are specific for lepidopteran species and MON 95379 maize would not be expected to adversely impact NTOs beneficial to agriculture at levels expected to be encountered in the field. As MON 95379 maize will not be commercialized in the U.S., a threatened and endangered species assessment focused on lepidopteran species in the states of Nebraska, Hawaii, and Iowa, where MON 95379 will be cultivated for small-scale breeding, testing, and seed increase activities and small-scale, confined field trials potentially in other locations to collect needed regulatory data to support global regulatory submissions of future products stacked with MON 95379. Results of the assessment support a conclusion of no effect for currently listed lepidopteran species.

- MON 95379 will not be commercialized in the U.S. and therefore will not impact U.S. commercial maize agricultural practices. It will only be cultivated in small-scale breeding, testing, and seed increase nurseries in the U.S. with typical production practices to produce high quality seed and/or data. Evaluation of the agronomic and phenotypic characteristics of MON 95379 support that, with the exception of the introduced trait, MON 95379 maize is phenotypically similar to conventional maize and is not expected to have an effect on maize agronomic practices under its intended use.

Maize is a Familiar Crop Lacking Weedy Characteristics

Maize is grown extensively throughout the world and is the largest cultivated grain crop followed by wheat (*Triticum* sp.) and rice (*Oryza sativa* L.) in total global production. In the U.S., maize is grown in almost all states and is the largest crop grown in terms of net value. Maize has been studied extensively, and the initial steps in its domestication can be traced back to approximately 9,000 years ago in southern Mexico. Although grown extensively throughout the world, maize is not considered a threat to invade natural or agricultural ecosystems because it does not establish self-sustaining populations outside of cultivation. This lack of weediness may reflect its poor competitive ability, lack of seed dormancy, and barriers to seed dispersal, as maize cobs retain seed and are covered in a husk. Several other characteristics common in weeds, such as rapid flowering following emergence, are lacking in maize. Traits often associated with weediness are typically not selected for during domestication and subsequent breeding and selection, and similarly, the history of maize breeding and production in the U.S. does not indicate there are any changes in the characteristics of maize that would increase the weediness of the crop. Although maize seed can overwinter in a rotation with soybeans or other crops, mechanical and chemical measures are routinely used to control maize volunteers. Maize is not sexually compatible with plant species occurring in the U.S. other than teosinte, an introduced wild relative. However, gene introgression from maize into teosinte is unlikely in the U.S. due to barriers to crossing, including morphological and developmental differences and limited geographical distribution of teosinte populations.

Conventional Maize LH44 is an Appropriate Comparator to MON 95379

Conventional control materials developed for use as comparators in safety assessment studies were based on the type of study conducted and the genetic background of the test material. The conventional control materials included the original transformation line (LH244) and LH244 crossed to a conventional line (HCL617) to create F1 starting control materials. LH244 was used as the control in molecular characterization studies. LH244 × HCL617 was used as the control in compositional analysis studies and in phenotypic, agronomic and environmental interactions assessments. Where appropriate, commercial hybrid maize materials (reference hybrids) were also used to establish a range of variability or responses representative of commercial maize in the U.S.

Molecular Characterization Verified the Integrity and Stability of the Inserted DNA in MON 95379

MON 95379 was produced by *Agrobacterium*-mediated transformation of maize tissue using the transformation vector PV-ZMIR522223. This vector contains a single T-DNA (transfer DNA), that is delineated by Right and Left Border regions. The T-DNA contains the *cry1B.868* and

cry1Da_7 expression cassettes. The T-DNA that was inserted initially contained a *cp4 epsps* selectable marker cassette flanked by two excision targeting sequences called *lox* sites. After MON 95379 was selected as an acceptable transformant, the selectable marker cassette was excised by crossing MON 95379 with a Cre recombinase expressing line (the "Cre line" was transformed with the vector PV-ZMOO513642). Subsequently, traditional breeding, segregation, selection, and screening were used to isolate those plants that contained the *cry1B.868* and *cry1Da_7* expression cassettes, and lacked the *cp4 epsps* selectable marker and any sequences from the *cre* gene containing plasmid, PV-ZMOO513642.

Characterization of the DNA insert in MON 95379 was conducted using a combination of sequencing, polymerase chain reaction (PCR), and bioinformatics. The results of this characterization demonstrate that MON 95379 contains one copy of the intended T-DNA containing the *cry1B.868* and *cry1Da_7* expression cassettes that is stably inherited over multiple generations and segregates according to Mendelian principles. These conclusions are based on several lines of evidence:

- Molecular characterization of MON 95379 by Next Generation Sequencing (NGS) demonstrated that MON 95379 contained a single intended DNA insert. These whole-genome analyses provided a comprehensive assessment of MON 95379 to determine the presence and identity of sequences derived from PV-ZMIR522223 and demonstrated that MON 95379 contained a single T-DNA insert, no detectable backbone or *cp4 epsps* selectable marker sequence from PV-ZMIR522223 or any sequences from PV-ZMOO513642.

- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) performed on MON 95379 was used to determine the complete sequence of the single DNA insert from PV-ZMIR522223, the adjacent flanking genomic DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the sequence and organization of the DNA is identical to the corresponding region in the PV-ZMIR522223 T-DNA and lacks the *cp4 epsps* selectable marker.

- Furthermore, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 95379 to the sequence of the insertion site in conventional maize. This analysis determined there was a 160 bp deletion upon T-DNA integration in MON 95379.

- Generational stability analysis by NGS demonstrated that the single PV-ZMIR522223 T-DNA insert in MON 95379 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 95379.

- Segregation analysis corroborates the insert stability demonstrated by NGS and independently establishes the nature of the T-DNA as a single chromosomal locus that shows an expected pattern of inheritance.

Taken together, the characterization of the genetic modification in MON 95379 demonstrates that a single copy of the intended T-DNA was stably integrated at a single locus of the maize genome

and that no PV-ZMIR522223 plasmid backbone, selectable marker, or PV-ZMOO513642 sequences are present in MON 95379.

Data Confirms Cry1B.868 and Cry1Da_7 Protein Safety

A multistep approach was used to characterize and assess the safety of the MON 95379 Cry1B.868 and Cry1Da_7 proteins. The expression level of the Cry1B.868 and Cry1Da_7 proteins in selected tissues of MON 95379 was determined and exposure to humans and other mammals through diet was evaluated. In addition, the donor organism for the *cry1B.868* and *cry1Da_7* coding sequences, *Bacillus thuringiensis*, is ubiquitous in the environment and is not commonly known for human or mammalian pathogenicity or allergenicity. Bioinformatics analysis determined that the Cry1B.868 and Cry1Da_7 proteins lack structural similarity to known allergens, gliadins, glutenins, or protein toxins which could have adverse effect to human or mammalian health. The Cry1B.868 and Cry1Da_7 proteins are rapidly digested in simulated digestive fluids and demonstrate no acute oral toxicity in mice at the levels tested that far exceed anticipated exposure by humans and mammals. Hence, the consumption of the Cry1B.868 and Cry1Da_7 proteins from MON 95379 or its progeny poses no risk to human and mammalian health.

MON 95379 is Compositionally Equivalent to Conventional Maize

Safety assessments of biotechnology-derived crops include a comparative safety assessment in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Maize is not known to have any endogenous toxicants or anti-nutrients associated with overall plant pest potential.

Compositional analysis was conducted on grain and forage of MON 95379 grown at five sites representative of typical agricultural regions for maize production in the U.S. in 2018. The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients and anti-nutrients in grain and forage of MON 95379 and the conventional control and followed considerations relevant to the compositional quality of maize as defined by the OECD consensus document (OECD, 2002a). Grain and forage samples were analyzed for levels of key nutrients including proximates, carbohydrates by calculation, fiber, amino acids, and linoleic acid. In addition, grain samples were analyzed for anti-nutrient levels of phytic acid and raffinose. Key nutrients in forage samples were analyzed for levels of protein, total fat, ash, carbohydrates by calculation, and fiber. In total, 25 different components were assayed (6 in forage and 19 in grain).

The results of the compositional assessment found that there were no compositional differences that were biologically meaningful between MON 95379 and conventional control and support the conclusion that MON 95379 maize is compositionally equivalent to the conventional control. These results support the overall food and feed safety of MON 95379.

MON 95379 Will Not Negatively Affect NTOs Including Those Beneficial to Agriculture

An evaluation of the impacts of MON 95379 on non-target organisms (NTOs) beneficial to agriculture is a component of a comprehensive environmental assessment. MON 95379 maize will not be commercialized in the U.S. and small-scale breeding, testing, and seed increase activities would be limited to a maximum total of 100 acres per growing season across three states (Nebraska, Hawaii and Iowa) as proposed in an EPA seed increase registration application, and small-scale, confined field trials potentially in other locations to collect needed regulatory data to support global regulatory submissions of future products stacked with MON 95379. Therefore, exposure of populations of non-target organisms will be low due to limited acreage and geography.

Prior to conducting a risk assessment for MON 95379 maize to beneficial NTOs, the proposed areas for cultivation of MON 95379 maize, the mode-of-action (MOA) and the spectrum of insecticidal activity of Cry1B.868 and Cry1Da_7 proteins, as well as expression analyses of the Cry1B.868 and Cry1Da_7 proteins produced by MON 95379 tissues were evaluated. The results of the activity spectrum studies indicate that, similar to previously commercialized Cry1 proteins, activity of Cry1B.868 and Cry1Da_7 proteins is limited to lepidopteran insects and effects are not expected on NTOs beneficial to agriculture. An evaluation of potential off-crop exposure to MON 95379 pollen indicated that significant exposure to non-target lepidopteran species is not anticipated and therefore MON 95379 poses minimal risk to non-target Lepidoptera. Results from Tier I NTO testing demonstrated no effects on survival from exposure to the Cry1B.868 and Cry1Da_7 proteins at levels well above those found in MON 95379 maize and in the environment. Calculated margins of exposure (MOE) based on LC_{50} values and conservative environmental concentrations were >11 for all species tested with the exception of *C. maculata* where the MOE for Cry1Da_7 was >7, indicating low risk to NTOs at field expression levels. A threatened and endangered species assessment focused on listed Lepidoptera in the states of Nebraska, Hawaii, and Iowa, where MON 95379 will primarily be cultivated, resulted in the conclusion of no effect for currently listed lepidopteran species.

MON 95379 Does Not Change Maize Plant Pest Potential or Environmental Interactions

Plant pest potential of a biotechnology-derived crop is assessed from the basis of familiarity that the USDA recognizes as an important underlying concept in risk assessment. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant hybrid or variety whose biological properties and plant pest potential are well known. Familiarity considers the biology of the plant, the introduced trait, the receiving environment, and the interactions among these factors. This provides a basis for the comparative risk assessment between a biotechnology-derived plant and the conventional control. Thus, the phenotypic, agronomic, and environmental interaction assessment of MON 95379 included the genetically similar conventional control as a comparator. This evaluation used a weight of evidence approach and considered statistical differences between MON 95379 and the conventional control with respect to reproducibility, magnitude, and directionality. Comparison to a range of commercial references grown concurrently established the range of natural variability for maize, and provided a context from which to further evaluate any statistical differences. Assessments included laboratory evaluation of seed germination and dormancy characteristics, as well as phenotypic and agronomic characteristics and plant responses to abiotic stressors, diseases, and arthropod pests in the field. Results from the germination, phenotypic, agronomic, and

environmental interactions assessments indicated that MON 95379 does not possess enhanced weediness characteristics, increased susceptibility or tolerance to specific abiotic stressors, diseases, non-target arthropod pests, or characteristics that would confer an increased plant pest risk compared to conventional maize.

Deregulation of MON 95379 is Not Expected to Have Effects on Maize Agronomic Practices

MON 95379 was developed to provide growers in South America an additional, effective tool for controlling target lepidopteran pests, including those resistant to current *Bt* technologies. As MON 95379 will not be commercialized in the U.S, no impact on agronomic or cultivation practices used in commercial maize production are expected.

While MON 95379 (and possible stack products containing MON 95379) may be grown on small acreage breeding/seed increase nurseries and agronomic/regulatory testing trials in limited geographies, subject to an EPA seed increase registration and applicable BRS permits, the cultivation of MON 95379 is not expected to significantly change current maize agronomic practices under the intended use. This conclusion is supported by the assessment of phenotypic, agronomic, and environmental interactions of MON 95379 that included the assessment of seed germination and dormancy characteristics, as well as phenotypic and agronomic characteristics and plant responses to abiotic stressors, diseases, and arthropod pests in the field under a range of environmental conditions. Results demonstrated that MON 95379 is similar to conventional maize in its agronomic and phenotypic characteristics and susceptibility or tolerance to specific abiotic stressors, diseases, or non-lepidopteran arthropod pests. Therefore, no changes in agronomic practices are anticipated for the cultivation of MON 95379 compared to conventional maize, particularly those that can significantly impact plant diseases or pests or their management. Furthermore, maize breeding and seed production nurseries are typically closely managed following best practices to control weed and insect pests to produce high quality data and seed. Based on this assessment, the intended use of MON 95379 is not expected to result in changes or impacts to current maize agronomic practices.

Conclusion

Based on the data and information presented in this petition, it is concluded that MON 95379 maize is unlikely to pose a greater plant pest risk than conventional maize. Therefore, in acknowledgment of the limitations that the EPA sets on the seed increase registration for MON 95379, Bayer requests a determination from USDA-APHIS that MON 95379 and any progeny derived from crosses between MON 95379 and conventional maize or deregulated biotechnology-derived maize be granted nonregulated status under 7 CFR Part 340.

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ABBREVIATIONS AND DEFINITIONS[1]

aadA	Aminoglycoside-modifying enzyme, 3"(9) –*O*– nucleotidyltransferase from the transposon Tn*7*
Act	Actin
ADF	Acid Detergent Fiber
APHIS	Animal and Plant Health Inspection Service
aroA	5-enolpyruvylshikimate-3-phosphate synthetase gene from *Agrobacterium* sp. Strain CP4
ANOVA	Analysis of Variance
AOSA	Association of Official Seed Analysts
BIO	Biotechnology Innovation Organization
bp	Basepair
BRS	Biotechnology Regulatory Service
Bt	*Bacillus thuringiensis*
bw	Body Weight
CBI	Confidential Business Information
CEW	Corn Earworm
CFR	Code of Federal Regulations
COA	Certificate of Analysis
COMPARE	COMprehensive Protein Allergen REsource
CP4 EPSPS	*Agrobacterium tumefaciens* strain CP4, 5-enolpyruvlshikimate-3-phosphate synthase protein
Cry	Crystalline Proteins
Cry1	Subspecies of Cry Proteins
Cry1B.868	Cry1B.868 is a chimeric protein comprised of domains I and II from Cry1Be (*Bt*), domain III from Cry1Ca (*Bt* subsp. *aizawai*) and C-terminal protoxin domain from Cry1Ab (*Bt* subsp. *kurstaki*)
Cry1Da_7	Modified Cry1Da protein derived from *Bt* subsp. *aizawai*
CTP	Chloroplast Transit Peptide
CTAB	Hexadecyltrimethylammonium Bromide
d	Day(s)
DNA	Deoxyribonucleic Acid
DT_{50}	50% Dissipation Time
dw	Dry Weight

[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

EC_{50}	Median Effective Concentration
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EEC	Estimated Environmental Concentrations
ELISA	Enzyme-linked Immunosorbent Assay
ELN	Electronic Lab Notebook
EPA	Environmental Protection Agency
ETS	Excellence Through Stewardship
FAO	Food and Agriculture Organization
FAW	Fall Armyworm
FDA	Food and Drug Administration (U.S.)
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FIR	Food Intake Rate
FMOC	fluorenylmethyl chloroformate
FMV	Figwort Mosaic Virus
FOIA	Freedom of Information Act
fw	Fresh Weight
GE	Genetically Engineered
GLP	Good Laboratory Practice
HESI	Health and Environmental Sciences Institute
IAC	Immunoaffinity Chromatography
ICP	Insect Control Proteins
ISF	International Seed Foundation
kb	Kilobase
kDa	Kilodalton
lbs	Pounds
LC_{50}	Median Lethal Concentration
LC/MS	Liquid chromatography-mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
LTP	Lipid Transfer Protein
mg/kg	Milligram per Kilogram
MOA	Mode of Action
MOE	Margins of Exposure
mRNA	Messenger RNA
MMT	Million Metric Ton
MW	Molecular Weight
NCBI	National Center for Biotechnology Information
NDF	Neutral Detergent Fiber
NGS	Next Generation Sequencing
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effects Level
NOEC	No Observed Effect Concentration
NTO	Non-Target Organism
OECD	Organization for Economic Co-operation and Development

OPA	o-phthalaldehyde
OR	Origin of Replication
ORF	Open Reading Frame
OSL	Over Season Leaf
OSR	Over Season Root
OT	Optimum Temperature
PCR	Polymerase Chain Reaction
PEC	Predicted environmental concentration
PIPs	Plant Incorporated Protectants
PPA	Plant Protection Act
SCB	Sugarcane Borer
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
S.E.	Standard Error
ShkG	5-enolpyruvylshikimate-3-phosphate synthase from *Arabidopsis thaliana*
SOP	Standard Operating Procedure
SOT	Suboptimum Temperature
T-DNA	Transfer Deoxyribonucleic Acid
TIP	Tonoplast Intrinsic Protein
Tub	Tubulin
µg/g	Microgram per Gram
Ubq	Ubiquitin gene
U.S.	United States
USDA	United States Department of Agriculture
UTR	Untranslated Region
WHO	World Health Organization

I. RATIONALE FOR THE DEVELOPMENT OF MON 95379

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 (U.S.) Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. APHIS regulations at 7 CFR § 340.6, effective as of the date this petition was filed, provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article. Newly promulgated 7 CFR § 340 (issued May 18, 2020) states, in §340.1(c), that "All plants determined by APHIS to be deregulated pursuant to § 340.6 as that section was set forth prior to August 17, 2020 will retain their nonregulated status under these regulations." Because this petition was filed before October 1, 2021 (see 7 CFR 340.1(c)(2)), Bayer seeks deregulation pursuant to the regulations in effect prior to August 17, 2020.

Bayer is submitting this request to APHIS for a determination of nonregulated status for the new biotechnology-derived maize product, MON 95379, any progeny derived from crosses between MON 95379 and conventional maize, and any progeny derived from crosses of MON 95379 with biotechnology-derived maize that have previously been granted nonregulated status under 7 CFR Part 340 or determined in the future to not pose a plant pest risk via a regulatory status review or otherwise deemed to be exempt from 7 CFR Part 340.

I.B. Rationale for the Development of Lepidopteran-Protected Maize MON 95379

Lepidopteran-protected maize MON 95379 was developed to provide growers in South America with new options for protection against the feeding damage of targeted lepidopteran pests, including fall armyworm (FAW; *Spodoptera frugiperda*), sugarcane borer (SCB; *Diatraea saccharalis*) and corn earworm (CEW; *Helicoverpa zea*). The larval feeding behavior of these species typically limits the efficacy of synthetic insecticides by creating additional difficulties for the sprayed active ingredients to reach the insects (Burtet et al., 2017; Grimi et al., 2018; Reay-Jones, 2019). MON 95379 produces two insecticidal proteins, Cry1B.868 and Cry1Da_7, which protect against feeding damage caused by these lepidopteran pests. Cry1B.868 is a chimeric protein comprised of domains I and II from Cry1Be (*Bacillus thuringiensis, Bt*), domain III from Cry1Ca (*Bt* subsp. *aizawai*) and the C-terminal protoxin domain from Cry1Ab (*Bt* subsp. *kurstaki*). Cry1Da_7 is a modified Cry1Da protein derived from *Bt* subsp. *aizawai*.

Fall Armyworm is the main pest of maize in most of South America. In warmer regions such as Brazil, where maize can be grown during two growing seasons (summer crop and winter crop), FAW populations can build up considerably under conditions favoring multiple and overlapping generations (Omoto et al., 2016). Synthetic insecticides have been the historic management tactic against FAW in maize and other crops in South America. Resistance to several distinctive classes of insecticides have been documented in Brazil (Carvalho et al., 2013; Diez-Rodríguez and Omoto,

2001; Nascimento et al., 2016; Okuma et al., 2018). Therefore, in this challenging environment for FAW management, *Bt* maize has become highly adopted by growers for FAW management in South America. Within nine years of Brazil's first introduction of insect-protected *Bt* maize in 2008, ~85% of the cultivated maize contained one or more insect-protection traits that expressed *Bt* proteins (ISAAA, 2017). Due to the appearance of resistance in FAW, growers in South America are in need of new tools to protect against larval feeding damage caused by lepidopteran pests. Resistance to *Bt* traits has already been observed for FAW to the proteins Cry1F and Cry1Ab and SCB to the proteins Cry1F and Cry1A.105 in South America (Bernardi et al., 2015a; Farias et al., 2014; Omoto et al., 2016). Currently, Vip3Aa20 is the only fully effective *Bt* protein against FAW available in the market in many regions in South America. However, an allele that confers resistance of FAW to Vip3Aa20 was already identified in Brazilian field populations (Amaral et al., 2020; Bernardi et al., 2016; Bernardi et al., 2015b), and is indicative of an increasing potential of resistance developing to this protein in FAW. The receptors that Cry1B.868 and Cry1Da_7 proteins in MON 95379 bind are different from each other in FAW and from other commercially available *Bt* proteins such as Cry1F, Cry1A.105, Cry2Ab, and Vip3 indicating that they would provide enhanced durability of this *Bt* maize technology in the field against this economically important pest (Wang et al., 2019).

Bayer will not seek a commercial registration for MON 95379 from the EPA since it will not be commercialized in North America. Under the proposed terms of an EPA seed increase registration application, MON 95379 will be cultivated for small-scale breeding, testing, and seed increase nurseries in Nebraska, Hawaii, and Iowa with a maximum total acreage of 100 acres per growing season. These activities will support the development of future commercial products for South America. In the course of developing these products containing MON 95379, Bayer may also develop breeding stacks in which MON 95379 will be combined with other traits for commercialization outside the United States. To support subsequent global regulatory submissions of these stack products, from time to time, Bayer may conduct confined, small-scale field trials that will be planted in additional counties/states that are assessed under the standard USDA-BRS permitting process. Trials and trial material will be managed according to the conditions of applicable BRS permits and terms of an EPA seed increase registration, if applicable.

I.C. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology (USDA-APHIS, 1986), the responsibility for regulatory oversight of biotechnology-derived maize falls primarily on three U.S. agencies: U.S. Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and in the case of plant incorporated protectants (PIPs), the Environmental Protection Agency (EPA). Deregulation of MON 95379 by USDA constitutes only one component of the overall regulatory oversight and review of this product by the three relevant U.S. regulatory agencies.

I.C.1. Submission to U.S. EPA

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) [7 U.S.C. §136(u)], are subject to regulation by U.S. EPA. Pesticides produced *in planta*, referred to as PIPs, are also subject to regulation by U.S. EPA under FIFRA.

Bayer submitted an application to U.S. EPA on May 1, 2020 requesting a FIFRA Section 3 seed increase registration (PRIA code B884) of the plant-incorporated protectant *Bacillus thuringiensis* Cry1B.868 and Cry1Da_7 proteins and the genetic material (Vector PV-ZMIR522223) necessary for their production in MON 95379. A maximum total U.S. acreage of 100 acres per growing season for breeding and seed increase purposes in the states of Nebraska, Hawaii and Iowa was requested. Thus MON 95379 will not be commercialized in the U.S., and Bayer will not seek a commercial registration (Section 3) for MON 95379 from the U.S. EPA.

In addition, a petition for a permanent exemption from the requirement of a tolerance for the plant-incorporated protectant (PIP) proteins Cry1B.868 and Cry1Da_7 in or on the food and feed commodities of maize was also submitted.

I.C.2. Submission to U.S. FDA

MON 95379 falls within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (U.S. FDA, 1992). In compliance with this policy, Bayer will initiate a formal consultation with the FDA and will be submitting a Food/Feed Safety and Nutritional Assessment Summary document to FDA in the near future.

I.C.3. Submissions to Foreign Government Agencies

Consistent with our commitments to the Biotechnology Innovation Organization (BIO) and Excellence Through Stewardship® (ETS)[2], Bayer will meet applicable regulatory requirements for MON 95379 in the country(ies) of intended production and for key import countries identified in a trade assessment process that have functioning regulatory systems to assure global compliance and support the flow of international trade. Bayer will continue to monitor other countries that are key importers of maize from the intended commercial production countries, for the development of new formal biotechnology approval processes. If new functioning regulatory processes are developed, Bayer will re-evaluate its stewardship plans and make appropriate modifications to minimize the potential for trade disruption.

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[2] http://www.excellencethroughstewardship.org/.

II. THE BIOLOGY OF MAIZE

The biology of maize has been well documented (Anderson and de Vicente, 2010; Farnham et al., 2003; OGTR, 2008). In addition to the wealth of information on maize biology, the Organisation for Economic Co-operation and Development authored a Consensus Document (OECD, 2003) on the biology of maize. This document provides key information regarding:

- general description of maize biology, including taxonomy and morphology and use of maize as a crop plant
- agronomic practices in maize cultivation
- geographic centers of origin
- reproductive biology
- cultivated maize as a volunteer weed
- inter-species/genus introgression into relatives and interactions with other organisms
- a summary of the ecology of maize

Additional information on the biology and uses of maize has also been compiled by the Australian Office of the Gene Technology Regulator (OGTR, 2008), and the Canadian Food Inspection Agency (CFIA, 2020). The taxonomic information for maize is available in the USDA's PLANTS Profile (USDA-NRCS, 2019).

To support the evaluation of the plant pest potential of MON 95379 relative to conventional maize, additional information regarding several aspects of maize biology can be found elsewhere in this petition. This includes: agronomic practices for maize in Section IX; volunteer management of maize in Section IX.B and inter-species/genus introgression potential in Section X.D.

II.A. Maize as a Crop

Maize is widely grown in nearly all areas of the world and is the largest grain crop in the world, ahead of both wheat (*Triticum sp.*) and rice (*Oryza sativa* L.), in total metric ton production (FAOSTAT, 2020). In the 2019/2020 marketing year, world maize area was approximately 192 million hectares (ha) with a total grain production of approximately 1,115 million metric tons (MMT) (USDA-FAS, 2020). The top five production regions were: USA (347 MMT), China (261 MMT), Brazil (101 MMT), the European Union (67 MMT), and Argentina (50 MMT) (USDA-FAS, 2020). In the U.S., maize is grown in most states and in 2019, its production value of over $53 billion was the highest of any crop (USDA-NASS, 2020).

In industrialized countries maize has two major uses: (1) as animal feed in the form of grain, forage or silage; and (2) as a raw material for wet- or dry-milled processed products such as high fructose maize syrup, oil, starch, glucose, dextrose and ethanol. By-products of the wet- and dry- mill processes are also used as animal feed. These processed products are used as ingredients in many industrial applications and in human food products. Most maize produced in industrialized countries is used as animal feed or for industrial purposes, but maize remains an important food staple in many developing regions, especially sub-Saharan Africa and Central America, where it is frequently the mainstay of human diets (Morris, 1998).

Maize is a familiar plant that has been rigorously studied due to its use as a staple food/feed and the economic opportunity it brings to growers. Archaeological and genetic evidence suggests that maize domestication began in southern Mexico approximately 9,000 years ago, and that it was derived from Balsas teosinte, *Zea mays* subsp. *parviglumis* (Kistler et al., 2018; Matsuoka et al., 2002; Piperno et al., 2009). Although grown extensively throughout the world, maize is not considered a threat to invade natural or agricultural ecosystems. Maize does not establish self-sustaining populations outside of cultivation (Crawley et al., 2001; OECD, 2003; Raybould et al., 2012). This lack of weediness may reflect its poor competitive ability (Olson and Sander, 1988), lack of seed dormancy, and barriers to seed dispersal, as maize cobs retain seed and are covered in a husk (Wilkes, 1972). A number of other characteristics common in weeds, such as rapid flowering following emergence, are lacking in maize (Keeler, 1989). Today, the majority of U.S. maize acreage is planted to hybrids, a practice that started in the 1920s (Wych, 1988). Maize hybrids have advantages in yield and plant vigor associated with heterosis, also known as hybrid vigor (Duvick, 1999).

Conventional plant breeding results in selection of desirable characteristics in a plant through the generation of unique combinations of genes obtained by intra- and inter-specific crossing, mutation breeding or utilization of other traditional breeding methodologies. However, there is a limit to the genetic diversity that is available for use and selection with conventional plant breeding. Biotechnology, as an additional tool to conventional breeding, offers access to greater genetic diversity than conventional breeding alone, resulting in expression of traits that are highly desirable to growers and downstream crop users.

II.B. Characteristics of the Recipient Plant

The MON 95379 transformation was conducted with inbred maize line LH244, a maize line assigned to Holden's Foundation Seeds, LLC in 2001 (U.S. Patent #6,252,148). LH244 is a medium season yellow dent maize line of Stiff Stalk background that is best adapted to the central regions of the U.S. corn belt.

Following transformation of immature LH244 embryos, selected transformants were self-pollinated to increase seed supplies. A Cre recombination system was used to remove the selectable marker starting with the selected events at the R2 generation. A selected inbred line homozygous for the presence of the T-DNA and lacking the selectable marker cassette was used to produce other MON 95379 materials for product testing, safety assessment studies, and commercial hybrid development. The non-transformed LH244 was used to produce conventional maize comparators (hereafter referred to as conventional controls) in the safety assessment of MON 95379.

II.C. Maize as a Test System in Product Safety Assessment

In studies utilizing hybrid maize, the test was a hybrid of LH244 containing MON 95379 (expressing the Cry1B.868 and Cry1Da_7 proteins) x HCL617 unless otherwise noted (Figure IV-4). LH244 x HCL617 was used as near isogenic, conventional control for the hybrid studies conducted (hereafter referred to as conventional control). LH244 was used as the conventional control in molecular characterization studies. LH244 x HCL617 was used as the conventional control in compositional analysis and in phenotypic, agronomic and environmental interactions assessments. Where appropriate, conventional commercial maize hybrids (hereafter referred to as reference hybrids) were used to establish ranges of natural variability or responses representative of commercial maize hybrids. Reference hybrids used at each field trial location were selected based on their availability and agronomic fit for the respective geographic regions.

To conduct the studies reported in this petition, appropriate MON 95379 test materials were generated for the molecular characterization (Sections III and IV), protein characterization and expression analysis (Section V), compositional analysis (Section VI), assessment of potential impacts on non-target organisms beneficial to agriculture and threatened and endangered (Section VII) and phenotypic, agronomic and environmental interactions assessment (Section VIII). The molecular characterization studies and initiation of commercial breeding efforts were conducted with the F4 generation (Figure IV-4). Protein characterization and expression analysis, composition analysis, and phenotypic, agronomic and environmental interactions assessment were conducted with MON 95379 breeding generation F5F1.

III. DESCRIPTION OF THE GENETIC MODIFICATION

This section provides a description of the transformation process and plasmid vector used in the development of MON 95379 (Figure III-1 and Figure III-2). Molecular analyses are an integral part of the characterization of maize products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into a single locus in the plant genome. This helps ensure that only the intended DNA encoding the desired trait(s) is integrated into the plant genome and facilitates the molecular characterization of the product. Information provided here allows for the identification of the genetic material present in the transformation vector delivered to the host plant and an analysis of the data supporting the characterization of the DNA inserted in the plant found in Section V.

III.A. Description of Transformation Plasmid PV-ZMIR522223

Plasmid Vector PV-ZMIR522223 was used for the transformation of conventional maize to produce MON 95379 and its plasmid map is shown in Figure III-2. A description of the genetic elements and their prefixes (e.g., B, P, TS, CS, T, I, E, and OR) in PV-ZMIR522223 is provided in Table III-1. PV-ZMIR522223 is approximately 21.6 kb and contains one T-DNA (transfer DNA), that is delineated by Right and Left Border regions. The T-DNA contains the *cp4 epsps* expression cassette, *cry1B.868* expression cassette, and the *cry1Da_7* expression cassette. During transformation, the T-DNA was inserted into the maize genome. Following transformation, traditional breeding, Cre/*lox* recombination (described in Section III.A), segregation, molecular screening and selection were used to isolate those plants that contained the *cry1B.868* and *cry1Da_7* expression cassettes and did not contain the backbone sequences from the transformation vector, the *cp4 epsps* selectable marker or any sequence from the *cre* gene-containing vector PV-ZMOO513642.

The *cry1B.868* coding sequence in MON 95379 is under the regulation of the promoter, 5' untranslated region (UTR) and intron for a ubiquitin gene (*Ubq*) from *Zea mays* subsp. *Mexicana* (Mexican teosinte) that directs transcription in plant cells (Cornejo et al., 1993). The *cry1B.868* coding sequence also utilizes the 3′ UTR sequence of the *Lipid Transfer Protein-like* gene (*LTP*) from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).

The codon optimized *cry1Da_7* coding sequence in MON 95379 is under the regulation of the promoter and 5' UTR from *Setaria italica* (foxtail millet) *tonoplast membrane integral protein* (*Tip*) gene (Hernandez-Garcia and Finer, 2014). In addition to the promoter and 5' UTR, the *cry1Da_7* coding sequence is regulated by the enhancer from the 35S RNA of figwort mosaic virus (FMV) (Richins et al., 1987) that enhances transcription in most plant cells (Rogers, 2000) and the intron and flanking UTR sequence from the *Actin 15* (*Act 15*) gene from *Oryza sativa* (rice) that is involved in regulating gene expression (Rose, 2008). The *cry1Da_7* coding sequence also utilizes 3' UTR sequence from the *GOS2* gene encoding a translation initiation factor from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).

The *cp4 epsps* selectable marker cassette is also part of the originally inserted T-DNA in MON 95379. The selectable marker cassette is under the regulation of the promoter, 5' UTR, intron and 3′ UTR sequences of the *OsTubA* gene family from *Oryza sativa* (rice) encoding

α-tubulin (Jeon et al., 2000) that directs transcription and polyadenylation of mRNA in plant cells. The *cp4 epsps* expression cassette was excised from progeny plants using the Cre/*lox* recombination system for marker removal as described in Section III.A.1 (Hare and Chua, 2002; Zhang et al., 2003).

The backbone region of PV-ZMIR522223, located outside of the T-DNA, contains two origins of replication for maintenance of the plasmid vector in bacteria (*oripBR322* and *ori V*) and a bacterial selectable marker gene (*aadA*).

III.A.1. Marker Removal Through Cre/*lox* Recombination System

The use of the Cre/*lox* recombination system for marker removal has been previously described (Hare and Chua, 2002; Russell et al., 1992; Zhang et al., 2003). The Cre/*lox* recombination system is derived from the bacteriophage P1 and consists of the Cre recombinase and a stretch of DNA flanked by two copies of the *lox*P sites. The *lox*P site is 34bp in length and consists of two 13-bp inverted repeats and an asymmetrical 8-bp spacer. The 13-bp inverted repeats are the Cre recombinase binding sequence, and the 8-bp spacer is essential for the recombination reaction. Cre recombinase binds to the inverted repeat sequence in the *lox*P site, catalyzing a crossover in the 8-bp spacer regions of the two *lox*P sites. The results of this crossover are two-fold: one is the excision of the DNA fragment flanked by the two half *lox*P sites (in MON 95379 this is the *cp4 epsps* selectable marker cassette) forming a circular extra-genomic DNA fragment, the other is the recombination of linear DNA between the remaining two half *lox*P sites within the maize genome (Gilbertson, 2003).

As reviewed (Gilbertson, 2003), one of the advantages of the Cre/*lox* system is the specificity of the enzyme for the wild-type *lox*P 34-bp recognition sequence. The frequency of Cre recombinase-mediated DNA recombination can be significantly reduced with even a single nucleotide change in specific regions of the *lox*P sequence (Hartung and Kisters-Woike, 1998; Hoess et al., 1986; Lee and Saito, 1998). Therefore, neither the specific DNA insert nor the usage of the Cre/*lox* system was expected to negatively influence the stability of the T-DNA in MON 95379 across breeding generations, which has been confirmed, and is described in Section IV.E. of this petition. This technology was previously reviewed by USDA in the petition for LY038 maize (USDA-APHIS petition #04-229-01p).

A maize line expressing Cre recombinase (developed with the transformation plasmid vector PV-ZMOO513642) was crossed with lines transformed with PV-ZMIR522223. In the resulting hybrid plants, the *cp4 epsps* selectable marker cassette that was flanked by the *lox*P sites was excised. The excised *cp4 epsps* selectable marker cassette (circular extra-genomic DNA) was subsequently not maintained during cell division. The *cre* gene and associated genetic elements were subsequently segregated away from the *cry1B.868* and *cry1Da_7* expression cassettes by conventional breeding to produce the MON 95379 product lacking the *cp4 epsps* gene cassette. The absence of both the *cp4 epsps* gene and *cre* gene were confirmed in the F4 generation (Figure IV-4). Since the *cp4 epsps* gene and sequence derived from PV-ZMOO513642 were eliminated through conventional breeding, the resulting progeny only contain the genes of interest (*cry1B.868* and c*ry1Da_7*) and not the gene used for selection.

III.B. Description of the Transformation System

MON 95379 was developed through *Agrobacterium tumefaciens* mediated transformation of immature maize embryos based on the method described by Sidorov and Duncan (2009) utilizing PV-ZMIR522223. Immature embryos were excised from a post-pollinated maize ear of LH244. After co-culturing the excised immature embryos with *Agrobacterium* carrying the plasmid vector, the immature embryos were placed on selection medium containing glyphosate and carbenicillin disodium salt in order to inhibit the growth of untransformed plant cells and excess *Agrobacterium*, respectively. Once transformed callus developed, the callus was placed on media conducive to shoot and root development. The rooted plants (R0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment. As demonstrated in this petition, the use of disarmed *Agrobacterium tumefaciens* strain ABI, a designated plant pest, as the transformation vector has not imparted plant pest characteristics to MON 95379.

The R0 plants were self-pollinated to produce R1 seed. Subsequently, the R1 population was screened for the presence of T-DNA and absence of vector backbone sequences by construct-level PCR assay and Southern blot analysis. Only plants that were homozygous positive for T-DNA and negative for vector backbone were selected for further development and their progenies were subjected to further molecular and phenotypic assessments. As is typical of a commercial event production and selection process, hundreds of different transformation events (regenerants) were generated in the laboratory using PV-ZMIR522223. Selected R2 events were crossed with a line expressing the Cre recombinase protein and screened for the absence of *cp4 epsps* and the *cre* genes. After careful selection and evaluation of these events in the laboratory, greenhouse and field, MON 95379 was selected as the lead event based on superior trait efficacy, agronomic, phenotypic, and molecular characteristics according to the general process described in Prado et al. (2014). Studies on MON 95379 were initiated to further characterize the genetic insertion and the expressed product, and to establish the food, feed, and environmental safety relative to conventional maize. The major steps involved in the development of MON 95379 are depicted in Figure III-1. The result of this process was the production of MON 95379 maize with the *cry1B.868* and c*ry1Da_7* expression cassettes.

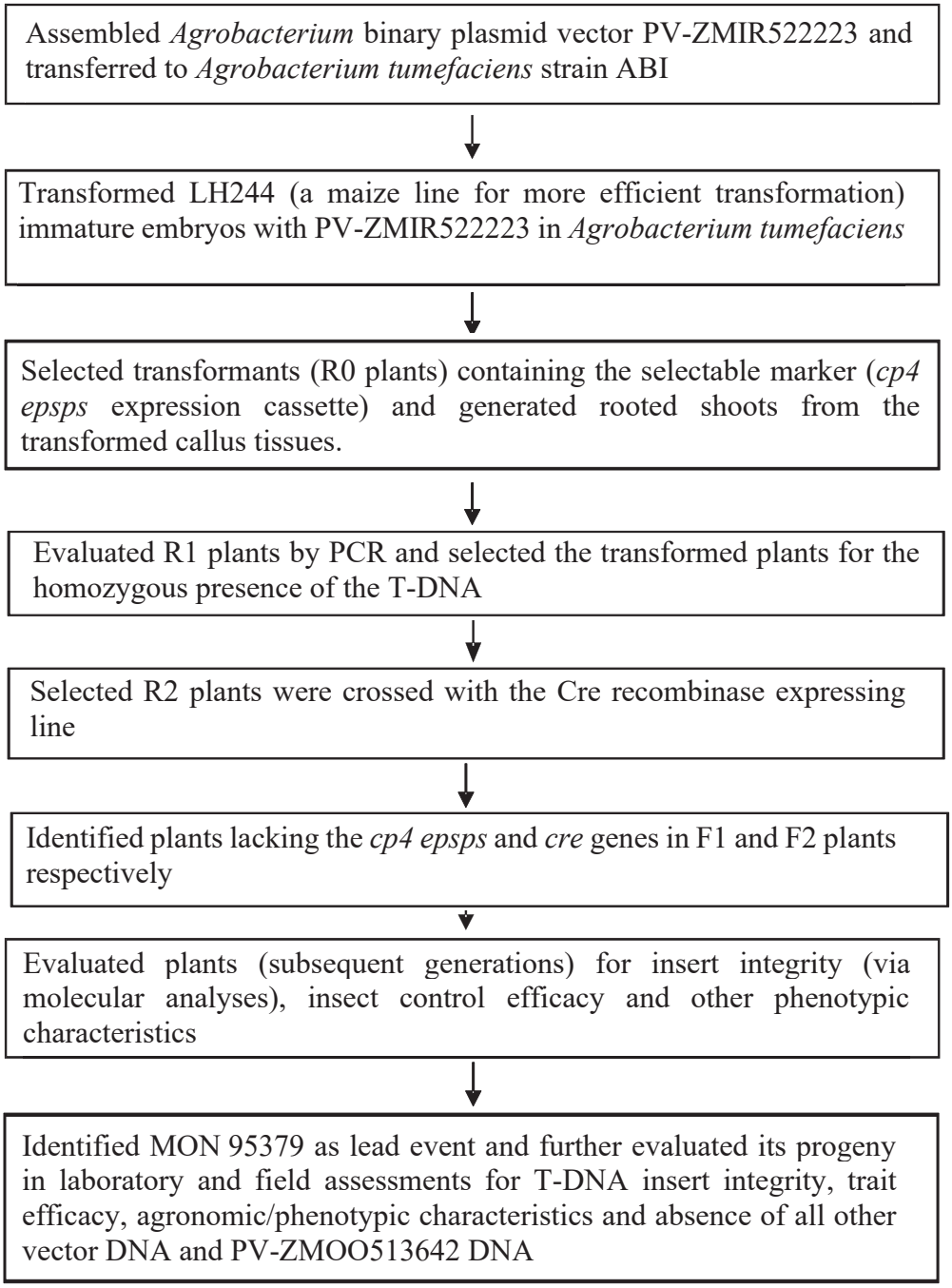

Figure III-1. Schematic of the Development of MON 95379

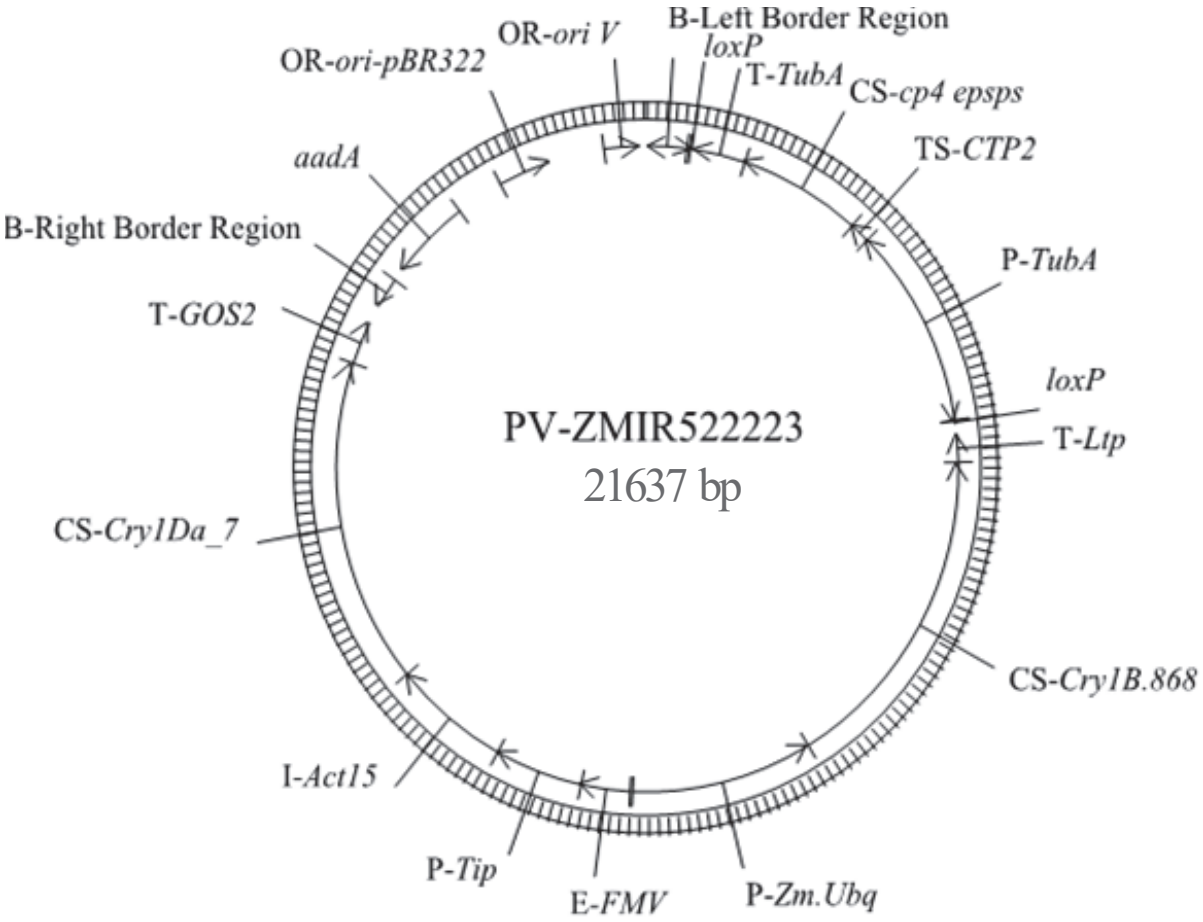

Figure III-2. Circular Map of PV-ZMIR522223

A circular map of PV-ZMIR522223 used to develop MON 95379 is shown. PV-ZMIR522223 contains one T–DNA. Genetic elements are shown on the exterior of the map.

Table III-1. Summary of Genetic Elements in Plasmid Vector PV-ZMIR522223

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T–DNA (Barker et al., 1983).
Intervening Sequence	443-477	Sequence used in DNA cloning
***lox*P**	478-511	Sequence from Bacteriophage P1 for the *lox*P recombination site recognized by the Cre protein (Russell et al., 1992).
Intervening Sequence	512-517	Sequence used in DNA cloning
T[4]-*TubA*	518-1099	3' UTR sequence of the *OsTubA* gene from *Oryza sativa* (rice) encoding α–tubulin (Jeon et al., 2000) that directs polyadenylation of mRNA.
Intervening Sequence	1100-1106	Sequence used in DNA cloning
CS[3]-*cp4 epsps*	1107-2474	Coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein that provides herbicide tolerance (Barry et al., 2001; Padgette et al., 1996).
TS[5]-*CTP2*	2475-2702	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee et al., 1987).
Intervening Sequence	2703-2706	Sequence used in DNA cloning
P[2]-*TubA*	2707-4887	Promoter, 5' UTR and intron sequences of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al., 2000) that directs transcription in plant cells.
Intervening Sequence	4888-4893	Sequence used in DNA cloning
***lox*P**	4894-4927	Sequence from Bacteriophage P1 for the *lox*P recombination site recognized by the Cre protein (Russell et al., 1992).

Table III-1. Summary of Genetic Elements in Plasmid Vector PV-ZMIR522223 (Continued)

Intervening Sequence	4928-5038	Sequence used in DNA cloning
T[4]-*Ltp*	5039-5338	3' UTR sequence of a *Lipid Transfer Protein-like* gene (*LTP*) from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).
Intervening Sequence	5339-5347	Sequence used in DNA cloning
CS[3]-*Cry1B.868*	5348-8947	Coding sequences of three domains and a protoxin sourced from *Bacillus thuringiensis* (*Bt*) parental proteins arranged as a single chimeric pesticidal protein (Cry1B.868) that confers protection against lepidopteran insects via insect midgut disruption (Wang et al., 2019).
Intervening Sequence	8948-8973	Sequence used in DNA cloning
P[2]-*Zm.Ubq*	8974-10981	Promoter, 5' UTR and first intron sequences of the ubiquitin (*Ubq*) gene from *Zea mays* subsp. *Mexicana* (Mexican teosinte) that directs transcription in plant cells (Cornejo et al., 1993).
Intervening Sequence	10982-11008	Sequence used in DNA cloning
E[9]-*FMV*	11009-11545	Enhancer from the 35S RNA of *Figwort mosaic virus* (FMV) (Richins et al., 1987) that enhances transcription in most plant cells (Rogers, 2000).
Intervening Sequence	11546-11556	Sequence used in DNA cloning
P[2]-*Tip*	11557-12537	Promoter and 5' UTR sequences from *Setaria italica* (foxtail millet) *tonoplast membrane integral protein* (*Tip*) gene (Hernandez-Garcia and Finer, 2014).
Intervening Sequence	12538-12545	Sequence used in DNA cloning
I[7]-*Act15*	12546-13838	Intron and flanking UTR sequence from the *Actin 15* (*Act 15*) gene from *Oryza sativa* (rice) that is involved in regulating gene expression (Rose, 2008).
Intervening Sequence	13839-13856	Sequence used in DNA cloning

Table III-1. Summary of Genetic Elements in Plasmid Vector PV-ZMIR522223 (Continued)

CS[3]-*Cry1Da_7*	13857-17357	Codon optimized coding sequence of *cry1Da_7* from *Bacillus thuringiensis* (*Bt*) encoding a modified Cry1Da_7 protein that confers protection against lepidopteran insects via insect midgut disruption (Wang et al., 2019).
Intervening Sequence	17358-17373	Sequence used in DNA cloning
T[4]-*GOS2*	17374-17841	3' UTR sequence from the *GOS2* gene encoding a translation initiation factor from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).
Intervening Sequence	17842-18045	Sequence used in DNA cloning
B[1]-Right Border Region	18046-18376	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T–DNA (Depicker et al., 1982; Zambryski et al., 1982).
Vector Backbone		
Intervening Sequence	18377-18520	Sequence used in DNA cloning
aadA	18521-19409	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3"(9) *–O–* nucleotidyltransferase from the transposon Tn*7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	19410-19943	Sequence used in DNA cloning
OR[8]-*ori-pBR322*	19944-20532	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979).
Intervening Sequence	20533-21154	Sequence used in DNA cloning
OR[8]-*ori V*	21155-21551	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981).
Intervening Sequence	21552-21637	Sequence used in DNA cloning

[1] B, Border
[2] P, Promoter
[3] CS, Coding Sequence
[4] T, Transcription Termination Sequence
[5] TS, Targeting Sequence
[6] L, Leader
[7] I, Intron
[8] OR, Origin of Replication

[9] E, Enhancer

III.C. The cry1B.868 Coding Sequence and Cry1B.868 Protein

The *cry1B.868* expression cassette in MON 95379 encodes a 127 kDa Cry1B.868 protein consisting of a single polypeptide of 1199 amino acids (Figure III-3.). As is typical for plant-expressed proteins, the lead methionine of the Cry1B.868 protein is not present. The *cry1B.868* coding sequence is the coding sequence of domains I and II from Cry1Be, domain III from Cry1Ca, and the C-terminal domain from Cry1Ab from various subspecies of soil bacterium *B. thuringiensis.* The presence of Cry1B.868 protein in maize provides protection against lepidopteran pests.

```
   1 MTSNRKNENE IINALSIPAV SNHSAQMNLS TDARIEDSLC IAEGNNIDPF
  51 VSASTVQTGI NIAGRILGVL GVPFAGQIAS FYSFLVGELW PRGRDPWEIF
 101 LEHVEQLIRQ QVTENTRDTA LARLQGLGNS FRAYQQSLED WLENRDDART
 151 RSVLYTQYIA LELDFLNAMP LFAIRNQEVP LLMVYAQAAN LHLLLLRDAS
 201 LFGSEFGLTS QEIQRYYERQ VEKTREYSDY CARWYNTGLN NLRGTNAESW
 251 LRYNQFRRDL TLGVLDLVAL FPSYDTRVYP MNTSAQLTRE IYTDPIGRTN
 301 APSGFASTNW FNNNAPSFSA IEAAVIRPPH LLDFPEQLTI FSVLSRWSNT
 351 QYMNYWVGHR LESRTIRGSL STSTHGNTNT SINPVTLQFT SRDVYRTESF
 401 AGINILLTTP VNGVPWARFN WRNPLNSLRG SLLYTIGYTG VGTQLFDSET
 451 ELPPETTERP NYESYSHRLS NIRLISGNTL RAPVYSWTHR SADRTNTISS
 501 DSINQIPLVK GFRVWGGTSV ITGPGFTGGD ILRRNTFGDF VSLQVNINSP
 551 ITQRYRLRFR YASSRDARVI VLTGAASTGV GGQVSVNMPL QKTMEIGENL
 601 TSRTFRYTDF SNPFSFRANP DIIGISEQPL FGAGSISSGE LYIDKIEIIL
 651 ADATFEAESD LERAQKAVNE LFTSSNQIGL KTDVTDYHID QVSNLVECLS
 701 DEFCLDEKKE LSEKVKHAKR LSDERNLLQD PNFRGINRQL DRGWRGSTDI
 751 TIQGGDDVFK ENYVTLLGTF DECYPTYLYQ KIDESKLKAY TRYQLRGYIE
 801 DSQDLEIYLI RYNAKHETVN VPGTGSLWPL SAPSPIGKCA HHSHHFSLDI
 851 DVGCTDLNED LGVWVIFKIK TQDGHARLGN LEFLEEKPLV GEALARVKRA
 901 EKKWRDKREK LEWETNIVYK EAKESVDALF VNSQYDRLQA DTNIAMIHAA
 951 DKRVHSIREA YLPELSVIPG VNAAIFEELE GRIFTAFSLY DARNVIKNGD
1001 FNNGLSCWNV KGHVDVEEQN NHRSVLVVPE WEAEVSQEVR VCPGRGYILR
1051 VTAYKEGYGE GCVTIHEIEN NTDELKFSNC VEEEVYPNNT VTCNDYTATQ
1101 EEYEGTYTSR NRGYDGAYES NSSVPADYAS AYEEKAYTDG RRDNPCESNR
1151 GYGDYTPLPA GYVTKELEYF PETDKVWIEI GETEGTFIVD SVELLLMEE
```

Figure III-3. Deduced Amino Acid Sequence of the Cry1B.868 Protein

The amino acid sequence of the MON 95379 Cry1B.868 protein was deduced from the full-length coding nucleotide sequence present in PV-ZMIR522223 (See Table III-1 for more details). The lead methionine (boxed with solid line) of the Cry1B.868 protein produced in MON 95379 is cleaved *in vivo*.

III.D. The *cry1Da_7* Coding Sequence and Cry1Da_7 Protein

The *cry1Da_7* expression cassette in MON 95379 encodes a 132 kDa Cry1Da_7 protein consisting of a single polypeptide of 1166 amino acids (Figure III-4). The lead methionine of the Cry1Da_7 protein is cleaved *in vivo*. The *cry1Da_7* coding sequence is a modified version of the *cry1Da* gene in soil bacterium *Bacillus thuringiensis* that encodes the Cry1Da protein. The Cry1Da_7 protein expressed by MON 95379 is highly homologous (approximately 99.7% sequence similarity) to the amino acid sequence of wild-type Cry1Da from *B. thuringiensis*, with one amino acid addition (alanine in position 2) and three amino acid substitutions (S282V, Y316S, I368P). The presence of Cry1Da_7 protein in maize provides protection against lepidopteran pests.

```
   1 MAEINNQNQC VPYNCLSNPK EIILGEERLE TGNTVADISL GLINFLYSNF
  51 VPGGGFIVGL LELIWGFIGP SQWDIFLAQI EQLISQRIEE FARNQAISRL
 101 EGLSNLYKVY VRAFSDWEKD PTNPALREEM RIQFNDMNSA LITAIPLFRV
 151 QNYEVALLSV YVQAANLHLS ILRDVSVFGE RWGYDTATIN NRYSDLTSLI
 201 HVYTNHCVDT YNQGLRRLEG RFLSDWIVYN RFRRQLTISV LDIVAFFPNY
 251 DIRTYPIQTA TQLTREVYLD LPFINENLSP AAVYPTFSAA ESAIIRSPHL
 301 VDFLNSFTIY TDSLARSAYW GGHLVNSFRT GTTTNLIRSP LYGREGNTER
 351 PVTITASPSV PIFRTLSYPT GLDNSNPVAG IEGVEFQNTI SRSIYRKSGP
 401 IDSFSELPPQ DASVSPAIGY SHRLCHATFL ERISGPRIAG TVFSWTHRSA
 451 SPTNEVSPSR ITQIPWVKAH TLASGASVIK GPGFTGGDIL TRNSMGELGT
 501 LRVTFTGRLP QSYYIRFRYA SVANRSGTFR YSQPPSYGIS FPKTMDAGEP
 551 LTSRSFAHTT LFTPITFSRA QEEFDLYIQS GVYIDRIEFI PVTATFEAEY
 601 DLERAQKVVN ALFTSTNQLG LKTDVTDYHI DQVSNLVACL SDEFCLDEKR
 651 ELSEKVKHAK RLSDERNLLQ DPNFRGINRQ PDRGWRGSTD ITIQGGDDVF
 701 KENYVTLPGT FDECYPTYLY QKIDESKLKA YTRYQLRGYI EDSQDLEIYL
 751 IRYNAKHEIV NVPGTGSLWP LSVENQIGPC GEPNRCAPHL EWNPDLHCSC
 801 RDGEKCAHHS HHFSLDIDVG CTDLNEDLGV WVIFKIKTQD GHARLGNLEF
 851 LEEKPLLGEA LARVKRAEKK WRDKRETLQL ETTIVYKEAK ESVDALFVNS
 901 QYDRLQADTN IAMIHAADKR VHRIREAYLP ELSVIPGVNA AIFEELEERI
 951 FTAFSLYDAR NIIKNGDFNN GLLCWNVKGH VEVEEQNNHR SVLVIPEWEA
1001 EVSQEVRVCP GRGYILRVTA YKEGYGEGCV TIHEIENNTD ELKFNNCVEE
1051 EVYPNNTVTC INYTATQEEY EGTYTSRNRG YDEAYGNNPS VPADYASVYE
1101 EKSYTDRRRE NPCESNRGYG DYTPLPAGYV TKELEYFPET DKVWIEIGET
1151 EGTFIVDSVE LLLMEE
```

Figure III-4. Deduced Amino Acid Sequence of the Cry1Da_7 Protein

The amino acid sequence of the MON 95379 Cry1Da_7 protein was deduced from the full-length coding nucleotide sequence present in PV-ZMIR522223 (See Table III-1 for more details). The lead methionine (boxed with solid line) of the Cry1Da_7 protein produced in MON 95379 is cleaved *in vivo*.

III.E. Regulatory Sequences

The *cry1B.868* coding sequence in MON 95379 is under the regulation of the promoter, 5' untranslated region (UTR) and intron for a ubiquitin gene (*Ubq*) from *Zea mays* subsp. *Mexicana* (*Mexican teosinte*) that direct transcription in plant cells (Cornejo et al., 1993). The *cry1B.868* coding sequence also utilizes the 3′ UTR sequence of the *Lipid Transfer Protein-like* gene (*LTP*) from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).

The codon optimized *cry1Da_7* coding sequence in MON 95379 is under the regulation of the enhancer from the 35S RNA of *Figwort mosaic virus* (FMV) (Richins et al., 1987) that enhances transcription in most plant cells (Rogers, 2000). In addition to the enhancer the *cry1Da_7* coding sequence is regulated by the promoter and 5' UTR from *Setaria italica* (foxtail millet) *tonoplast membrane integral protein* (*Tip*) gene (Hernandez-Garcia and Finer, 2014) and the intron and flanking UTR sequence from the *Actin 15* (*Act 15*) gene from *Oryza sativa* (rice) that is involved in regulating gene expression (Rose, 2008). The *cry1Da_7* coding sequence also utilizes 3' UTR sequence from the *GOS2* gene encoding a translation initiation factor from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).

The *cp4 epsps* selectable marker cassette is also part of the originally inserted T-DNA in MON 95379. The selectable marker cassette is under the regulation of the promoter, 5' UTR, intron and 3′ UTR sequences of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al., 2000) that directs transcription and polyadenylation of mRNA in plant cells. The *cp4 epsps* gene was eliminated from subsequent progeny using the Cre/*lox* recombination system for marker removal (Hare and Chua, 2002; Zhang et al., 2003). The *cp4 epsps* cassette was flanked by *lox*P sites that allowed the cassette to be excised by Cre recombinase when plants were crossed with maize plants expressing the *cre* gene (the "Cre line" was developed with the transformation plasmid vector PV-ZMOO513642). The *cre* gene was subsequently segregated out by conventional breeding to produce the MON 95379 product from which the *cp4 epsps* gene was eliminated. The absence of both the *cp4 epsps* gene, the *cre* gene and their associated genetic elements were confirmed in the F4 generation (Figure IV-4). Since the *cp4 epsps* gene and sequence derived from PV-ZMOO513642 were eliminated through conventional breeding, the resulting progeny only contain the genes of interest (*cry1B.868* and *cry1Da_7*) and not the gene used for selection.

III.F. T-DNA Border Regions

PV-ZMIR522223 contains Left and Right Border regions (Figure III-2 and Table III-1) that were derived from *A. tumefaciens* plasmids. The border regions each contain a nick site that is the site of DNA exchange during transformation (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer of T-DNA into the maize genome.

III.G. Genetic Elements Outside the T-DNA Border Regions

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-ZMIR522223 in bacteria and are referred to as plasmid backbone. The origin of replication, *ori V*, is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host range plasmid RK2 (Stalker et al., 1981). The origin of replication, *ori-pBR322*, is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe, 1979). The selectable marker *aadA* is the coding sequence for an aminoglycoside-modifying enzyme, 3"(9)–*O*–nucleotidyltransferase from the transposon Tn*7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the maize genome. The absence of the backbone and other unintended plasmid sequence in MON 95379 was confirmed by sequencing and bioinformatic analyses (Section IV.B).

IV. CHARACTERIZATION OF THE GENETIC MODIFICATION

This section describes the methods and results of a comprehensive molecular characterization of the genetic modification present in MON 95379. It provides information on the DNA insertion(s) into the plant genome of MON 95379, and additional information regarding the arrangement and stability of the introduced genetic material.

IV.A. Description of Methodology Used to Characterize MON 95379

A schematic representation of the next generation sequencing (NGS) methodology and the basis of the characterization using NGS and PCR sequencing are illustrated in Figure IV-1 below. Appendix B defines the test, control and reference substances, and provides an additional overview of these techniques, their use in DNA characterization in maize plants and the materials and methods.

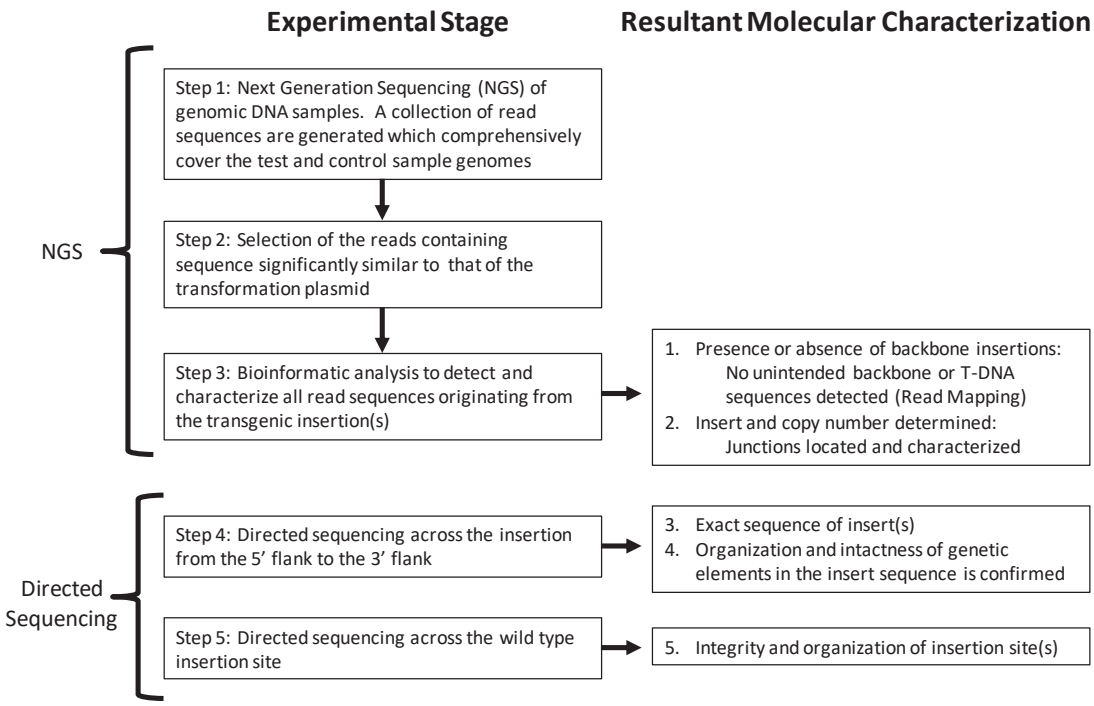

Figure IV-1. Molecular Characterization using Sequencing and Bioinformatics

Genomic DNA from the test and the conventional control was sequenced using technology that produces a set of short, randomly-distributed sequence reads that comprehensively cover test and control genomes (Step 1). Utilizing these genomic sequence reads, bioinformatics searches are conducted to identify all sequence reads that are significantly similar to the transformation plasmid (Step 2). These captured reads are then mapped and analyzed to determine the presence/absence of transformation plasmid backbone sequences, identify insert junctions, and to determine the insert and copy number (Step 3). Overlapping PCR products are also produced which span any insert and their wild type locus (Step 4 and Step 5, respectively); these overlapping PCR products are sequenced to allow for detailed characterization of the inserted DNA and insertion site.

The NGS method was used to characterize the genomic DNA from MON 95379 and the conventional control by generating short (~150 bp) randomly distributed sequence fragments (sequencing reads) generated in sufficient number to ensure comprehensive coverage of the sample genomes. It has been previously demonstrated that whole genome sequencing at 75× depth of coverage is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic et al., 2012). A comprehensive analysis of NGS as a characterization method demonstrated that coverage depth as low as 11× is sufficient to detect both intended transgenes as well as unintended inserted vector-derived fragments as small as 25 base pairs in length (Cade et al., 2018). Therefore, 75× coverage is a robust level of sequencing for the complete characterization of both homozygous and hemizygous transgenes, and, well in excess of the levels which have been demonstrated as sufficient for identifying unintended inserted fragments. To confirm sufficient sequence coverage of the genome, the 150 bp sequence reads were analyzed to determine the coverage of a known single-copy endogenous maize gene. This establishes the depth of coverage (the median number of times each base of the genome is independently sequenced). Furthermore, the sensitivity of the method was assessed by sequencing the transformation plasmid and then sampling the data to represent a single genome equivalent dataset and a $1/10^{th}$ genome equivalent dataset. This confirms the method's ability to detect any sequences derived from the transformation plasmid. Bioinformatics analysis was then used to select sequencing reads that contained sequences similar to the transformation plasmid, and these were analyzed in depth to determine the number of DNA inserts. NGS was run on five breeding generations of MON 95379 and the appropriate conventional controls. Results of NGS are shown in Sections IV.B and IV.E.

The DNA inserts of MON 95379 was determined by mapping of sequencing reads relative to the transformation plasmid and identifying junctions and unpaired read mappings adjacent to the junctions. Examples of five types of NGS reads are shown in Figure IV-2. The junctions of the DNA insert and the flanking DNA are unique for each insertion (Kovalic et al., 2012). Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions.

Directed sequencing (locus-specific PCR and DNA sequencing analyses, Figure IV-1, Step 4) complements the NGS method. Sequencing of the insert and flanking genomic DNA determined the complete sequence of the insert and flanks by evaluating if the sequence of the insert was identical to the corresponding sequence from the T-DNA in PV-ZMIR522223, and if each genetic element in the insert was intact. It also characterizes the flank sequence beyond the insert corresponding to the genomic DNA of the transformed maize. Results are described in Sections IV.B, IV.C and IV.D; methods are presented in Appendix B.

The stability of the T-DNA present in MON 95379 across multiple breeding generations was evaluated by NGS as described above. This information was used to determine the number and identity of the DNA inserts in each generation. For a single copy T-DNA insert, two junction sequence types are expected. In the case of an event where a single locus is stably inherited over multiple breeding generations, two identical junction sequences would be detected across all the breeding generations tested. Results are described in Section IV.E; methods are presented in Appendix B.

Segregation analysis of the T-DNA was conducted to determine the inheritance and generational stability of the insert in MON 95379. Segregation analysis corroborates the insert stability

demonstrated by NGS and independently establishes the genetic behavior of the T-DNA. Results are described in Section IV.F.

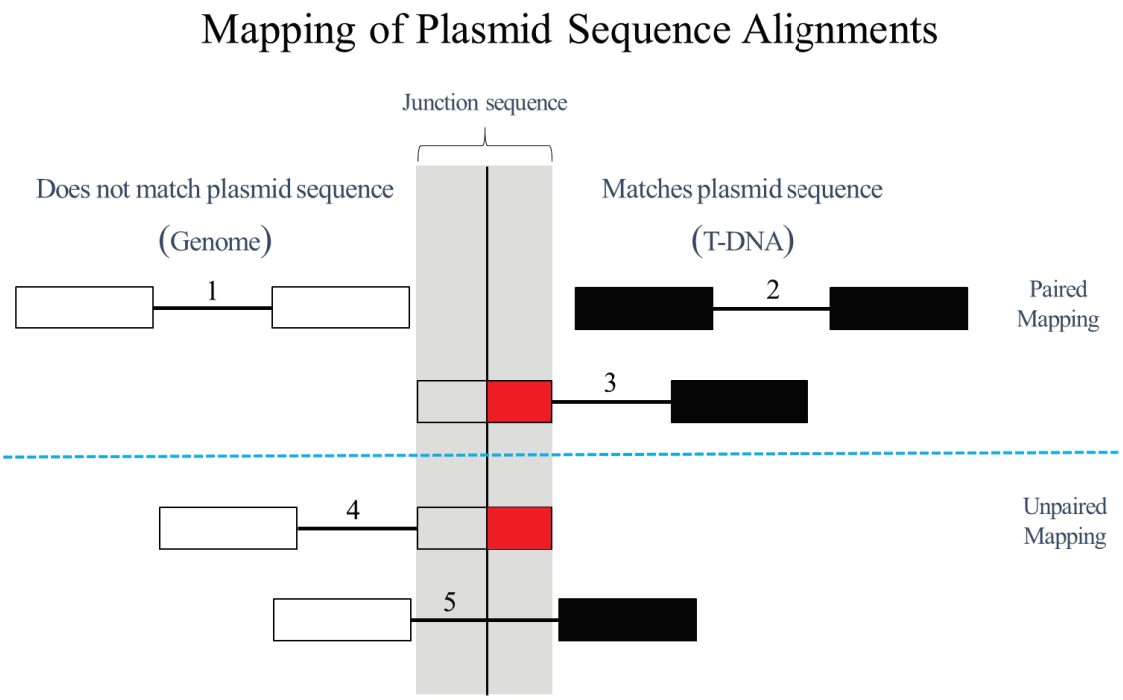

Figure IV-2. Five Types of NGS Reads

NGS yields data in the form of read pairs where sequence from each end of a size selected DNA fragment is returned. Depicted above are five types of sequencing reads/read pairs generated by NGS sequencing which can be found spanning or outside of junction points. Sequence boxes are color-filled if it matches with plasmid sequence, and empty if it matches with genomic sequence. Grey highlighting indicates sequence reads spanning the junction. Junctions are detected by examining the NGS data for reads having portions of plasmid sequences that span less than the full read, as well as reads mapping adjacent to the junction points where their mate pair does not map to the plasmid sequence. The five types of sequencing reads/read pairs being (1) Paired and unpaired reads mapping to genomic sequence outside of the insert, greater than 99.999% of collected reads fall into this category and are not evaluated in this analysis, (2) Paired reads mapping entirely to the transformation plasmid sequence, such reads reveal the presence of transformation sequence in planta, (3) Paired reads where one read maps entirely within the inserted DNA and the other read maps partially to the insert (indicating a junction point), (4) Single read mapping partially to the transformation plasmid DNA sequence (indicating a junction point) where its mate maps entirely to the genomic flanking sequence and (5) Single read mapping entirely to the transformation plasmid DNA sequence where its mate maps entirely to genomic flanking sequence, such reads are part of the junction signature.

IV.B. Characterization of the DNA Inserts in MON 95379

The number of inserted DNA sequences from PV-ZMIR522223 in MON 95379 was assessed by generating a comprehensive collection of reads via NGS of MON 95379 genomic DNA using the F4 generation (Figure IV-4). A plasmid map of PV-ZMIR522223 is shown in Figure III-2. Table IV-1 provides descriptions of the genetic elements present in MON 95379. A schematic representation of the insert and flanking sequences in MON 95379 is shown in Figure IV-3. For full details on materials and methods see Appendix B.

IV.B.1. Next Generation Sequencing for MON 95379 and Conventional Control Genomic DNA

Genomic DNA from five distinct breeding generations of MON 95379 (Figure IV-4) and conventional controls were isolated from seed and prepared for sequencing. For material and method details see Appendix B. These genomic DNA libraries were used to generate short (~150 bp) randomly distributed sequencing reads of the maize genome (Figure IV-1, Step 1).

To demonstrate sufficient sequence coverage the ~150 bp sequence reads were analyzed by mapping all reads to a known single copy endogenous gene (*Zea mays* pyruvate decarboxylase (*pdc3*), GenBank Accession: AF370006.2) in each of the five distinct breeding generations. The analysis of sequence coverage plots showed that the depth of coverage (i.e., the median number of times any base of the genome is expected to be independently sequenced) was 82× or greater for the five generations of MON 95379 (F4, F5, F4F1, F5F1, and F6F1) and the conventional controls (Appendix B, Table B-1). It has been previously demonstrated that whole genome sequencing at 75× depth of coverage provides comprehensive coverage and ensures detection of inserted DNA (Cade et al., 2018; Kovalic et al., 2012). Total sequencing generated, renderings of mappings across subsequent generations, controls, and reference substances, and summary statistics of T-DNA coverage are included in Appendix K.

To demonstrate the method's ability to detect any sequences derived from the PV-ZMIR522223 transformation plasmid or the Cre recombinase-containing transformation plasmid vector PV-ZMOO513642, a sample of PV-ZMIR522223 and PV-ZMOO513642, were sequenced by NGS following the same processes outlined for all samples in Appendix B. The resulting PV-ZMIR522223 and PV-ZMOO513642 reads were randomly selected to achieve a depth of one and $1/10^{th}$ genome equivalent (relative to the median coverage of the LH244 conventional control). In all cases, 100% coverage of the known PV-ZMIR522223 or PV-ZMOO513642 sequences were observed (Appendix B, Table B-2). This result demonstrates that all nucleotides of PV-ZMIR522223 and PV-ZMOO513642 are detectable by the sequencing and bioinformatic assessments performed and that a detection level of at least $1/10^{th}$ genome equivalent was achieved for the plasmid DNA sequence assessment.

Table IV-1. Summary of Genetic Elements in MON 95379

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
5' Flanking DNA	1-1000	DNA sequence flanking the 5' end of the insert
B[3]-Left Border Region r1	1001-1186	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T–DNA (Barker et al., 1983).
Intervening Sequence	1187-1221	Sequence used in DNA cloning
***lox*P**	1222-1255	Sequence from Bacteriophage *P1* for the *lox*P recombination site recognized by the Cre recombinase (Russell et al., 1992).
Intervening Sequence	1256-1366	Sequence used in DNA cloning
T[4]-*Ltp*	1367-1666	3' UTR sequence of a *Lipid Transfer Protein-like* gene (*LTP*) from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).
Intervening Sequence	1667-1675	Sequence used in DNA cloning
CS[5]-*Cry1B.868*	1676-5275	Coding sequences of three domains and a protoxin sourced from *Bacillus thuringiensis* (*Bt*) parental proteins arranged as a single chimeric pesticidal protein (Cry1B.868) that confers protection against lepidopteran insect pests via insect midgut disruption (Wang et al., 2019).
Intervening Sequence	5276-5301	Sequence used in DNA cloning
P[6]-*Zm.Ubq*	5302-7309	Promoter, 5' UTR and first intron sequences of the ubiquitin (*Ubq*) gene from *Zea mays* subsp. *Mexicana* (Mexican teosinte) that directs transcription in plant cells (Cornejo et al., 1993).
Intervening Sequence	7310-7336	Sequence used in DNA cloning
E[7]-*FMV*	7337-7873	Enhancer from the 35S RNA of figwort mosaic virus (FMV) (Richins et al., 1987) that enhances transcription in most plant cells (Rogers, 2000).
Intervening Sequence	7874-7884	Sequence used in DNA cloning

Table IV-1. Summary of Genetic Elements in MON 95379 (continued)

P-*Tip*	7885-8865	Promoter and 5' UTR sequences from *Setaria italica* (foxtail millet) *tonoplast membrane integral protein* (*Tip*) gene (Hernandez-Garcia and Finer, 2014).
Intervening Sequence	8866-8873	Sequence used in DNA cloning
I[8]-*Act15*	8874-10166	Intron and flanking UTR sequence from the *Actin 15* (*Act 15*) gene from *Oryza sativa* (rice) that is involved in regulating gene expression (Rose, 2008).
Intervening Sequence	10167-10184	Sequence used in DNA cloning
CS-*Cry1Da_7*	10185-13685	Codon optimized coding sequence of *cry1Da_7* from *Bacillus thuringiensis* (*Bt*) encoding a modified Cry1Da_7 protein that confers protection against lepidopteran insect pests via insect midgut disruption (Wang et al., 2019).
Intervening Sequence	13686-13701	Sequence used in DNA cloning
T-*GOS2*	13702-14169	3' UTR sequence from the *GOS2* gene encoding a translation initiation factor from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).
Intervening Sequence	14170-14322	Sequence used in DNA cloning
3' Flanking DNA	14323-15322	DNA sequence flanking the 3' end of the insert

[1] Although flanking sequences and intervening sequences are not functional genetic elements, they comprise a portion of the sequence.
[2] Numbering refers to the sequence of the insert in MON 95379 and adjacent DNA
[3] B, Border
[4] T, Transcription Termination Sequence
[5] CS, Coding Sequence
[6] P, Promoter
[7] E, Enhancer
[8] I, Intron
[r1] Superscript in Left indicate that the sequence in MON 95379 was truncated compared to the sequences in PV-ZMIR522223

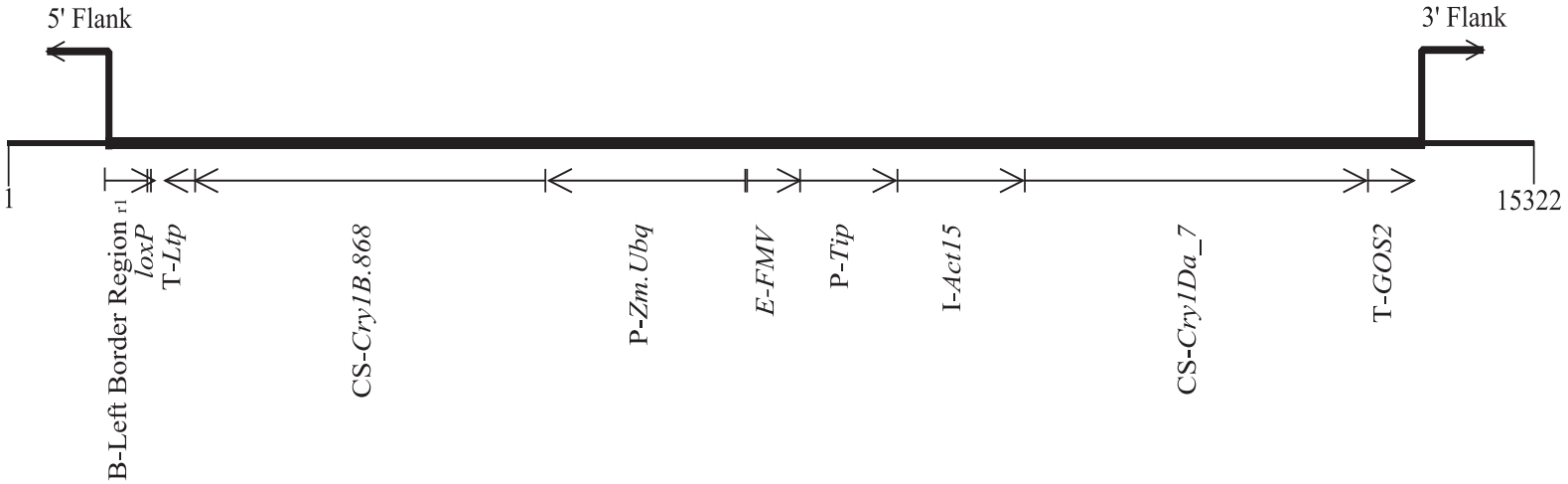

Figure IV-3. Schematic Representation of the Insert and Flanking Sequences in MON 95379

DNA derived from T-DNA of PV-ZMIR522223 integrated in MON 95379. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking sequence. Identified on the map are genetic elements within the insert. This schematic diagram may not be drawn to scale.

[r1] Superscript in Left Border Regions indicate that the sequence in MON 95379 was truncated compared to the sequences in PV-ZMIR522223

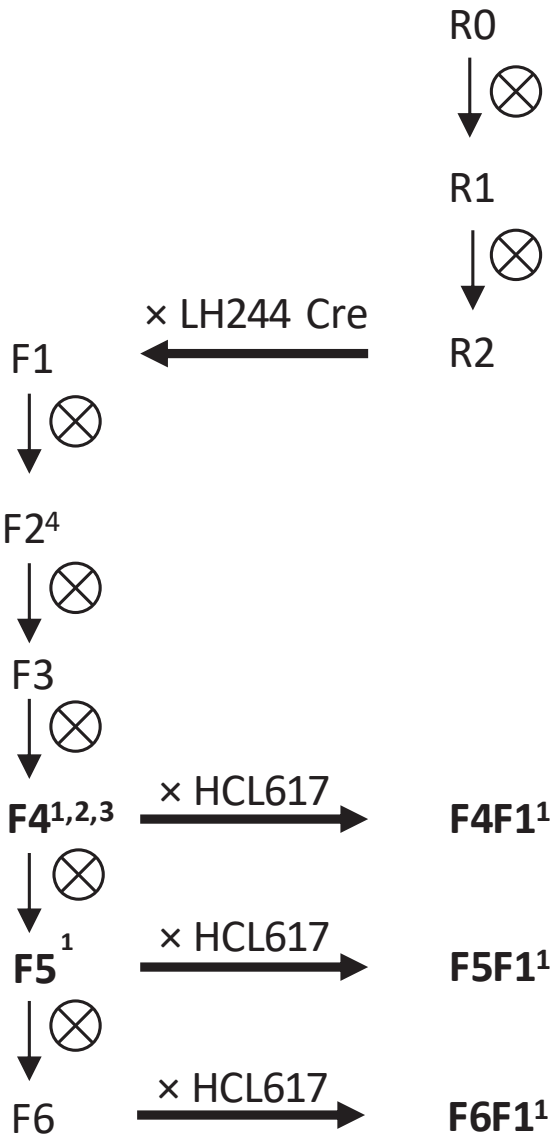

Figure IV-4. Breeding History of MON 95379

The generations used for molecular characterization and insert stability analyses are indicated in bold text. R0 corresponds to the transformed plant, ⊗ designates self-pollination.

[1]Generations used to confirm insert stability.
[2] Generation used for molecular characterization.
[3] Generation used for commercial development of MON 95379.
[4] The F2 generation was screened for plants lacking the *cre* gene. Only those plants lacking the *cre* gene were self-pollinated to create a F3 population of plants lacking the *cre* gene.

IV.B.2. Selection of Sequence Reads Containing Sequence of the PV-ZMIR522223 and PV-ZMOO513642

The transformation plasmid, PV-ZMIR522223, was transformed into the parental variety LH244 to produce MON 95379. Consequently, any DNA inserted into MON 95379 will consist of sequences that are similar to the PV-ZMIR522223 DNA sequence. Therefore, to fully characterize the DNA from PV-ZMIR522223 inserted in MON 95379, it is sufficient to completely analyze only the sequence reads that have similarity to PV-ZMIR522223 (Figure IV-1, Step 2). Similarly, to confirm the absence of the Cre-containing transformation plasmid vector, PV-ZMOO513642, in MON 95379 it is sufficient to completely analyze only the sequence reads that have similarity to PV-ZMOO513642.

Using established criteria (described in the materials and methods, Appendix B), any sequence reads similar to PV-ZMIR522223 and PV-ZMOO513642 were selected from MON 95379 sequence datasets (PV-ZMOO513642 selection was only conducted on the F4 generation) and were then used as input data for bioinformatic junction sequence analysis. PV-ZMIR522223 and PV-ZMOO513642 sequences were also compared against the conventional control sequence datasets.

IV.B.3. Determination of T-DNA Copy Number and Presence or Absence of Plasmid Vector Backbone

Mapping sequence reads relative to the transformation plasmid allows for the identification of junction signatures, the presence or absence of plasmid backbone sequence and the number of T-DNA insertions. For a single copy T-DNA insert sequence at a single genomic locus and the complete absence of plasmid vector backbone, a single junction signature pair and few if any reads aligning with the transformation plasmid backbone sequences are expected.

When reads from the LH244 dataset were aligned with the transformation plasmid sequence, a number of reads mapped to the T-DNA promoter element *Zm.Ubq* sequence (Figure IV-5). The alignment of these sequence reads is the result of an endogenous maize sequence that is homologous to the T-DNA encoded promoter element *Zm.Ubq* sequence. No other regions of homology were identified between the PV-ZMIR522223 transformation plasmid reference sequence and the conventional control.

When reads from the MON 95379 (F4) dataset were aligned with the transformation plasmid sequence, large numbers of reads mapped to the intended T-DNA sequence, one paired read was identified which aligned to the transformation plasmid backbone, a number of unpaired reads aligned to the T-DNA promoter element *Zm.Ubq*, and no reads mapped to the selectable marker cassette (Figure IV-6). The presence of unpaired reads aliging to this *Zm.Ubq* are indicative of sequences from an endogenous maize element mapping to the transformation vector (Appendix L). This is most readily observed by comparing and contrasting the unpaired reads aligning to the *Zm.Ubq* region to those observed in the LH244 conventional control dataset (Panel 1, Figures IV-5 and IV-6). A comparison of mapped reads from F4 and the conventional control reveals that mapped reads are present at sample equivalent levels and show virtually identical mapping signatures. This, in conjunction with an absence of junction sites, indicates that all additional reads and subsequent relative increases of depth across this region are entirely derived

from the endogenous maize *Zm.Ubq* homolog, and therefore not indicative of any additional T-DNA insertion (partial or whole). The mapping of large numbers of reads from the MON 95379 (F4) dataset to the intended T-DNA sequence is expected and fully consistent with the presence of the inserted DNA.

A single pair of reads was found to align with OR-*ori-pBR322* sequences (Figure IV-6). The sporadic low-level detection of plasmid sequences such as OR-*ori-pBR322* has previously been described (Zastrow-Hayes et al., 2015), and reported (see Supplemental Figure S1 in Yang et al. (2013)), and is due to the presence of environmental bacteria in tissue samples used in the preparation of genomic DNA used for library construction. The presence of this sequence from environmental bacteria does not indicate the presence of backbone sequence in the MON 95379 (F4) generation. This analysis indicates that MON 95379 (F4) does not contain inserted sequence from the transformation plasmid backbone.

No reads mapped to the selectable marker cassette (T-*TubA*, TS-*CTP2,* CS- *cp4 epsps*, and P-*TubA*). This result is expected as MON 95379 was crossed with a Cre recombinase expressing line that enable the removal of the *cp4 epsps* gene cassette positioned between two excision targeting *lox* sites (Hare and Chua, 2002; Zhang et al., 2003). After excision, a single *lox* site remained, as expected. The absence of the selectable marker cassette results in a gap in reads as illustrated in Figure IV-6.

To determine the insert number in MON 95379 (F4), selected reads mapping to T-DNA as described above were analyzed to identify junctions. This bioinformatic analysis is used to find and classify partially matched reads characteristic of the ends of insertions. The number of unique junctions determined by this analysis are shown in Table IV-2.

Table IV-2. Unique Junction Sequence Class Results

Sample	Junctions Detected
MON 95379 (F4)	2
LH244	0

Detailed mapping information of the junction sequences is shown in Figure IV-6. The location and orientation of the junction sequences relative to T-DNA insert determined for MON 95379 are illustrated in Figure IV-6, panels 1 and 2. As shown in the figure, there are two junctions identified in MON 95379. Both junctions contain the T-DNA border sequence joined to flanking genomic sequence, indicating that they represent the sequences at the junctions of the intended T-DNA insert and the maize genome. As described earlier, no junctions were detected in any of the conventional maize control samples.

Considered together, the absence of plasmid backbone and the presence of two junctions (joining T-DNA borders and flanking sequences) indicate a single intended T-DNA at a single locus in the genome of MON 95379. Both of these junctions originate from the same locus of the MON 95379 genome and are linked by contiguous, previously determined and expected DNA sequence (with the exception of the selectable marker cassette which was excised as described earlier). This is

demonstrated by complete coverage of the sequenced reads spanning the interval between the junctions and the directed sequencing of overlapping PCR products described in Section IV.C.

Based on the comprehensive NGS and junction identification it is concluded that MON 95379 contains one copy of the T-DNA inserted into a single locus. This conclusion is confirmed by the sequencing and analysis of overlapping PCR products from this locus as described in Section IV.C.

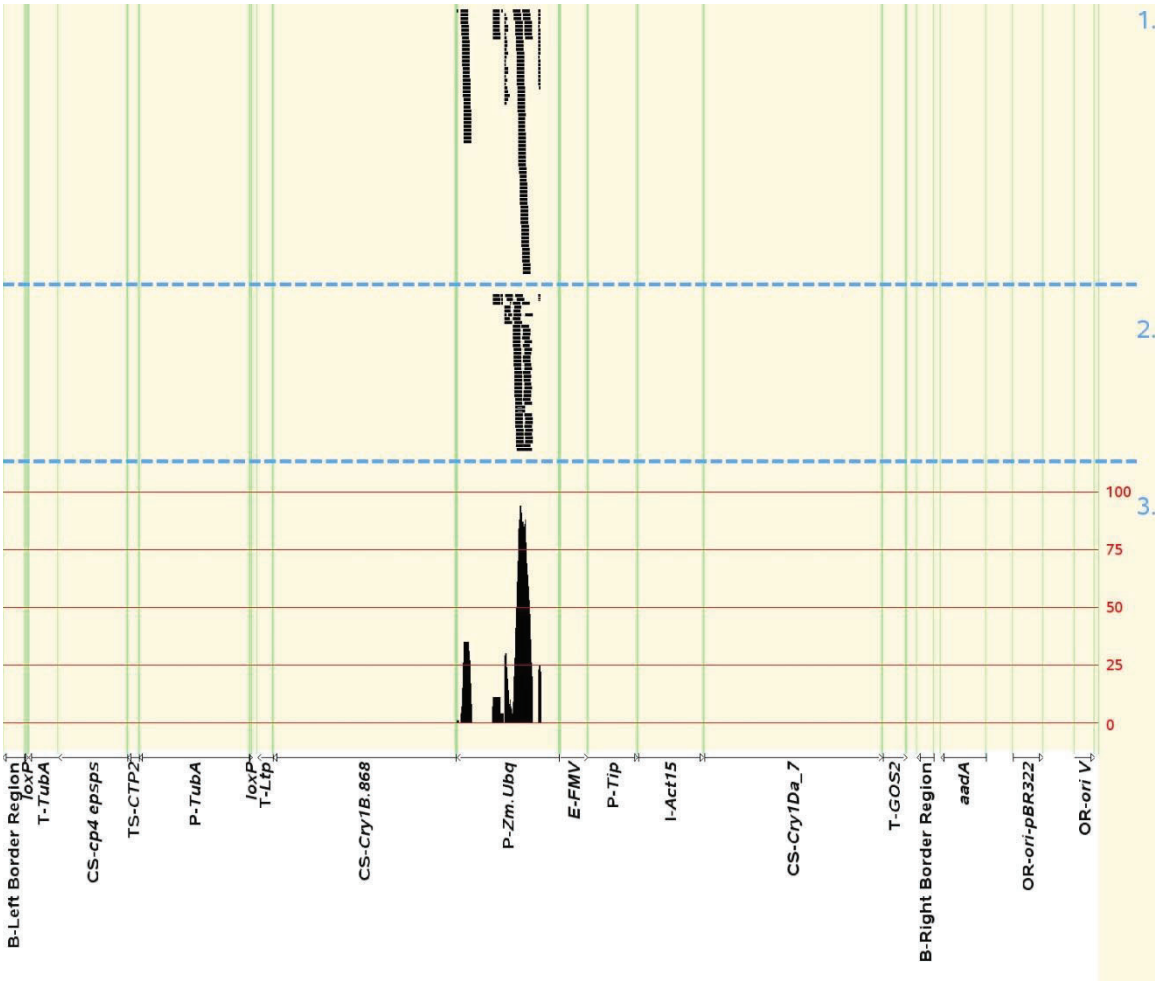

Figure IV-5. Read Mapping of Conventional Maize LH244 Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference.

Vertical lines show genetic element boundaries.

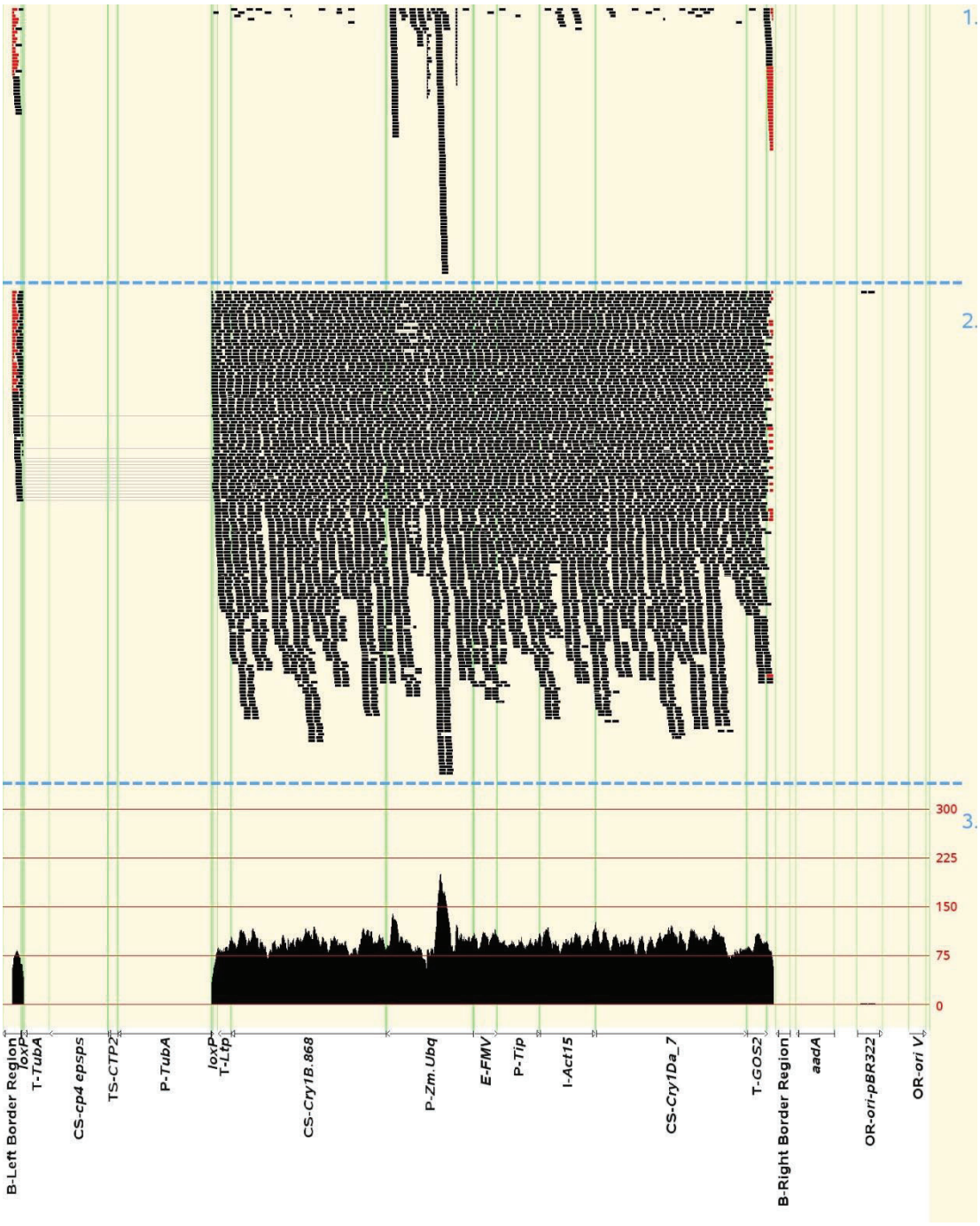

Figure IV-6. Read Mapping of MON 95379 (F4) Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads. Panel 2 shows paired mapped reads and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference sequence. Vertical lines show genetic element boundaries. The region of flank junction sequences that align with the transformation plasmid are shown in red. Comparable results were observed when read mapping the F4F1, F5, F5F1, and F6F1 generations of MON 95379 versus PV-ZMIR522223 (Appendix K).

IV.B.4. Determination of Absence of Plasmid Vector PV-ZMOO513642

At the R2 generation (Figure IV-4), MON 95379 was crossed with a Cre recombinase expressing line. The Cre/*lox* technology enables the removal of the selectable marker cassette (T-*TubA*, TS-*CTP2,* CS-*cp4 epsps*, and P-*TubA*) which was inserted during the transformation as part of the T-DNA insertion that also included the *cry1B.868* and *cry1Da_7* trait cassettes. The resulting F1 progeny were self-pollinated and plants from the F2 generation were screened for the absence of the *cre* gene and associated genetic elements (and other sequences from plasmid PV-ZMOO513642), allowing for selection of lines lacking the Cre recombinase cassette that were used as the progenitor of subsequent generations and the final product.

To confirm the absence of the Cre recombinase cassette and any part of the *cre* gene containing transformation vector, MON 95379 was assessed by utilizing the comprehensive collection of generation F4 NGS reads and subsequently mapping them to the *cre* gene containing transformation plasmid (PV-ZMOO513642) sequence Figure IV-7. In the absence of any PV-ZMOO513642 insertions there should be zero junction signature pairs and limited reads aligning with the PV-ZMOO513642 sequences.

When reads from the MON 95379 (F4) dataset were aligned with the PV-ZMOO513642 sequence, a number of reads aligned to the left border region (B-Left Border Region). Only a small number of reads mapping to the promoter element *Ract1* mapped to T-DNA, and only one paired read was identified which aligned to the PV-ZMOO513642 backbone (Figure IV-7).

The mapping of a number of reads from the MON 95379 (F4) dataset to the left border region (B-Left Border Region) is expected since PV-ZMOO513642 and PV-ZMIR522223 share the same left border region sequence and PV-ZMIR522223's left border region is present in MON 95379 (Figure IV-6). The small number of reads mapping to the promoter element *Ract1* are also present in the LH244 control background. This is fully consistent with the presence of a homologous sequence being present in the LH244 control background (Figure IV-8) and does not indicate the presence of PV-ZMOO513642 T-DNA in MON 95379. Additionally, a single pair of reads was found to align with OR-*ori-pBR322* sequences (Figure IV-7). The sporadic low level detection of plasmid sequences such as OR-*ori-pBR322* has previously been described (Zastrow-Hayes et al., 2015), and reported (see Supplemental Figure S1 in Yang et al. (2013)), and is due to the presence of environmental bacteria in tissue samples used in the preparation of genomic DNA used for library construction. As such, the incidence of this sequence from environmental bacteria does not indicate the presence of backbone sequence in the MON 95379 (F4) generation. Considered together, the limited reads aligning with the PV-ZMOO513642 sequences and the absence junctions (joining T-DNA borders and flanking sequences; Table IV-3) indicate that MON 95379 (F4), and subsequent generations, do not contain inserted sequence from the *cre* gene containing transformation plasmid, PV-ZMOO513642.

Table IV-3. Unique Junction Sequence Class Results

Sample	Junctions Detected
MON 95379 (F4)	0
LH244	0

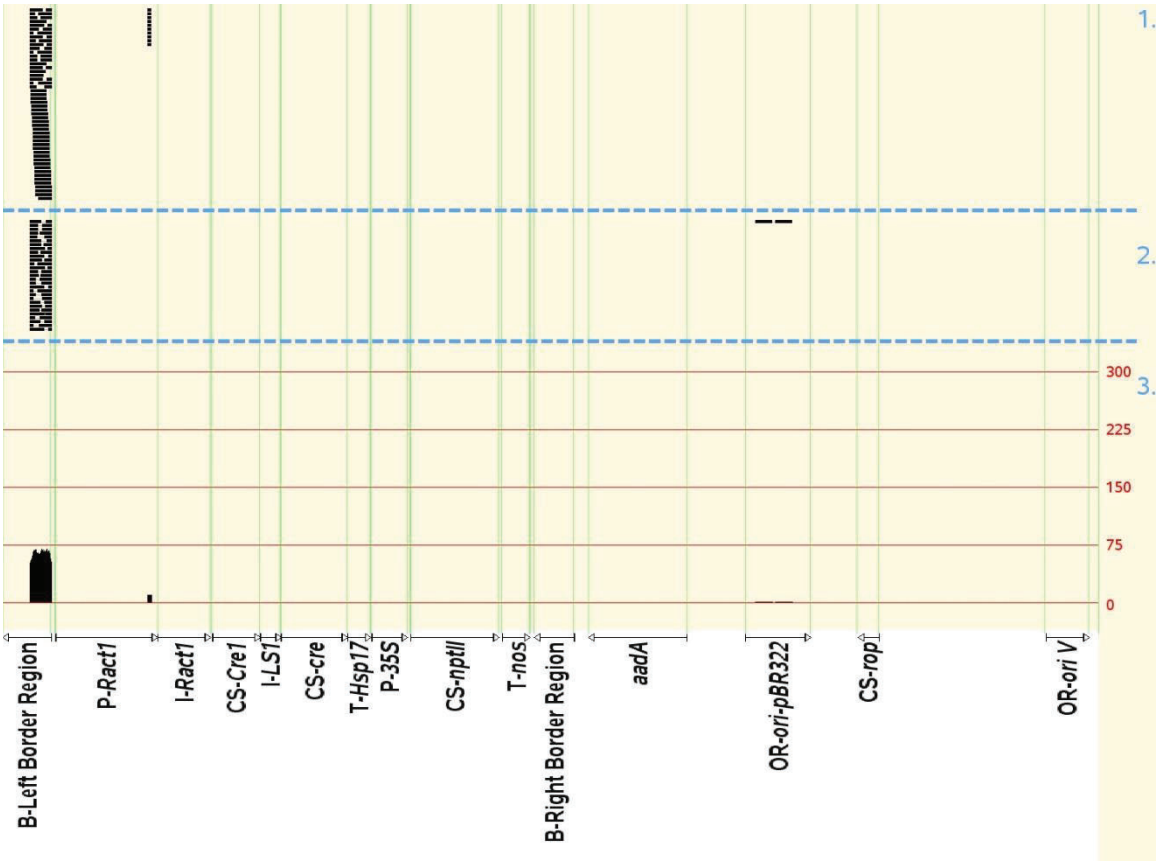

Figure IV-7. Read Mapping of MON 95379 (F4) Versus PV-ZMOO513642

Panel 1 shows the location of unpaired mapped reads. Panel 2 shows paired mapped reads and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference sequence. Vertical lines show genetic element boundaries.

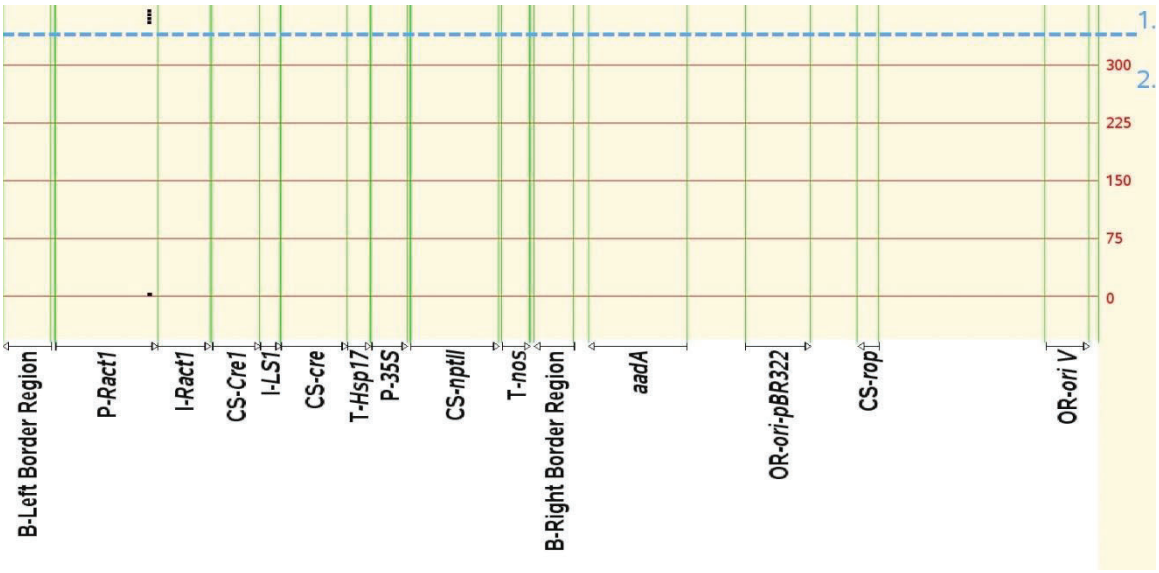

Figure IV-8. Read Mapping of Conventional Maize LH244 Versus PV-ZMOO513642

Panel 1 shows the location of unpaired mapped reads, there are no paired mapped reads, and Panel 2 shows a representation of combined raw read depth for unpaired and paired reads across the reference sequence.

Vertical lines show genetic element boundaries.

IV.C. Organization and Sequence of the Insert and Adjacent DNA in MON 95379

The organization of the elements within the DNA insert and the adjacent genomic DNA was assessed using directed DNA sequence analysis (refer to Figure IV-1, Step 4). PCR primers were designed to amplify two overlapping regions of the MON 95379 genomic DNA that span the entire length of the insert and the adjacent DNA flanking the insert (Figure IV-9). The amplified PCR products were subjected to DNA sequencing analyses. The results of this analysis confirm that the MON 95379 insert is 13322 bp and that each genetic element within the T-DNA is intact compared to PV-ZMIR522223, with the exception of the border regions. The right border region was absent and the left border region contained small terminal deletions with the remainder of the inserted border region being identical to the sequence in PV-ZMIR522223. The sequence and organization of the insert was also shown to be identical to the corresponding T-DNA of PV-ZMIR522223 as intended. As noted, in Section IV.B.3, the selectable marker cassette (T-*TubA*, TS-*CTP2,* CS-*cp4 epsps*, and P-*TubA*) and one *lox*P site (bases 478 through 4893 of the PV-ZMIR522223 sequence) were excised by Cre recombinase, and as expected, are not present in the MON 95379 sequence. This analysis also shows that only T-DNA elements (described in Table IV-1) were present. In addition, 1000 base pairs flanking the 5′ end of the MON 95379 insert (Table IV-1, bases 1-1000) and 1000 base pairs flanking the 3′ end of the MON 95379 insert (Table IV-1, bases 14323-15322) were determined.

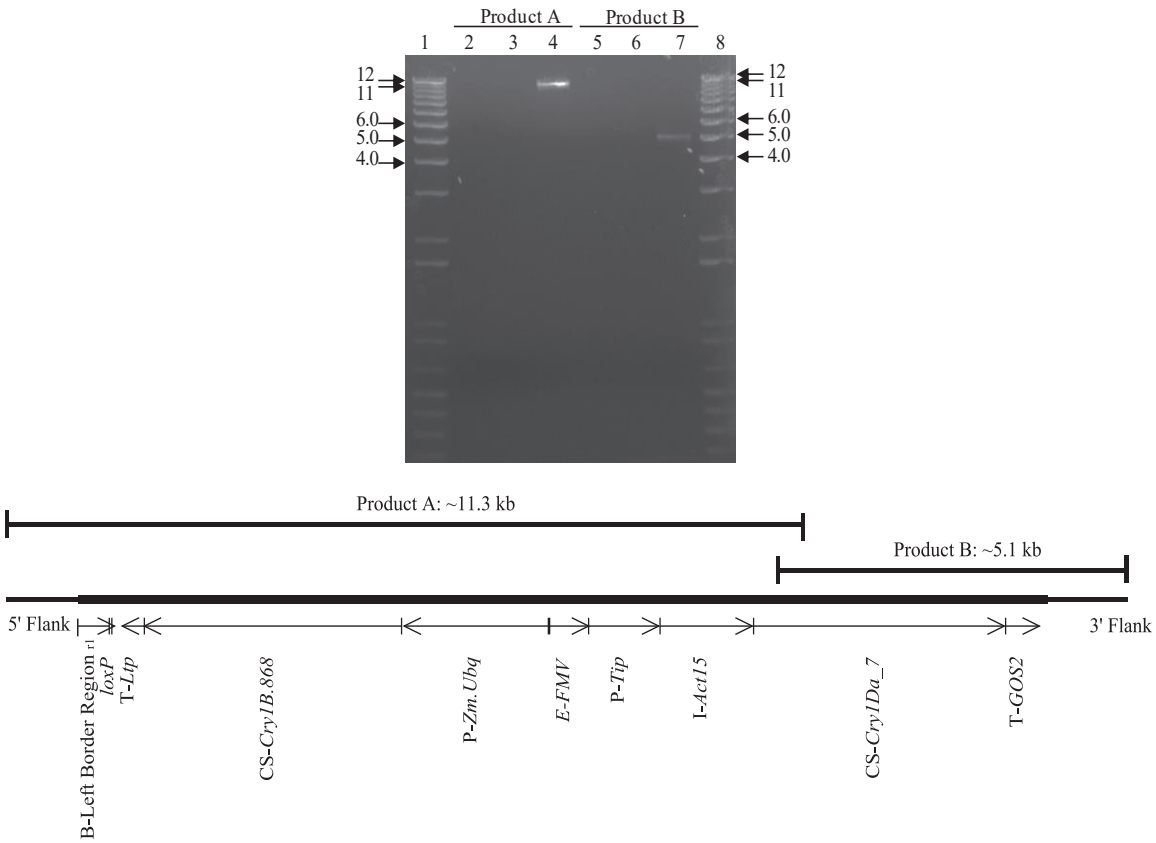

Figure IV-9. Overlapping PCR Analysis across the Insert in MON 95379

PCR was performed on both conventional control genomic DNA and genomic DNA of the F4 generation of MON 95379 using two pairs of primers to generate overlapping PCR fragments from MON 95379 for sequencing analysis. To verify the PCR products, 2 µl of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 95379 that appears at the bottom of the figure. This figure is a representative of the data generated in the study. Lane designations are as follows:

Lane	
1	1 Kb Plus DNA Ladder
2	No template control
3	LH244 Conventional Control
4	MON 95379
5	No template control
6	LH244 Conventional Control
7	MON 95379
8	1 Kb Plus DNA Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb Plus DNA Ladder (Invitrogen) on the ethidium bromide stained gel.

[r1] Superscript in Left Border Regions indicate that the sequence in MON 95379 was truncated compared to the sequences in PV-ZMIR522223.

IV.D. Sequencing of the MON 95379 Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from the conventional control to examine the insertion site in conventional maize (see Figure IV-1, Step 5). The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5' end of the MON 95379 insert paired with a second primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure IV-10). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5' and 3' flanking sequences of MON 95379 indicates that 160 bases of maize genomic DNA were deleted during integration of the T-DNA. Such changes are common during plant transformation (Anderson et al., 2016) and these changes presumably resulted from double stranded break repair mechanisms in the plant during *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998). The remainder of the maize genomic DNA sequences flanking the insert in MON 95379 are identical to the conventional control.

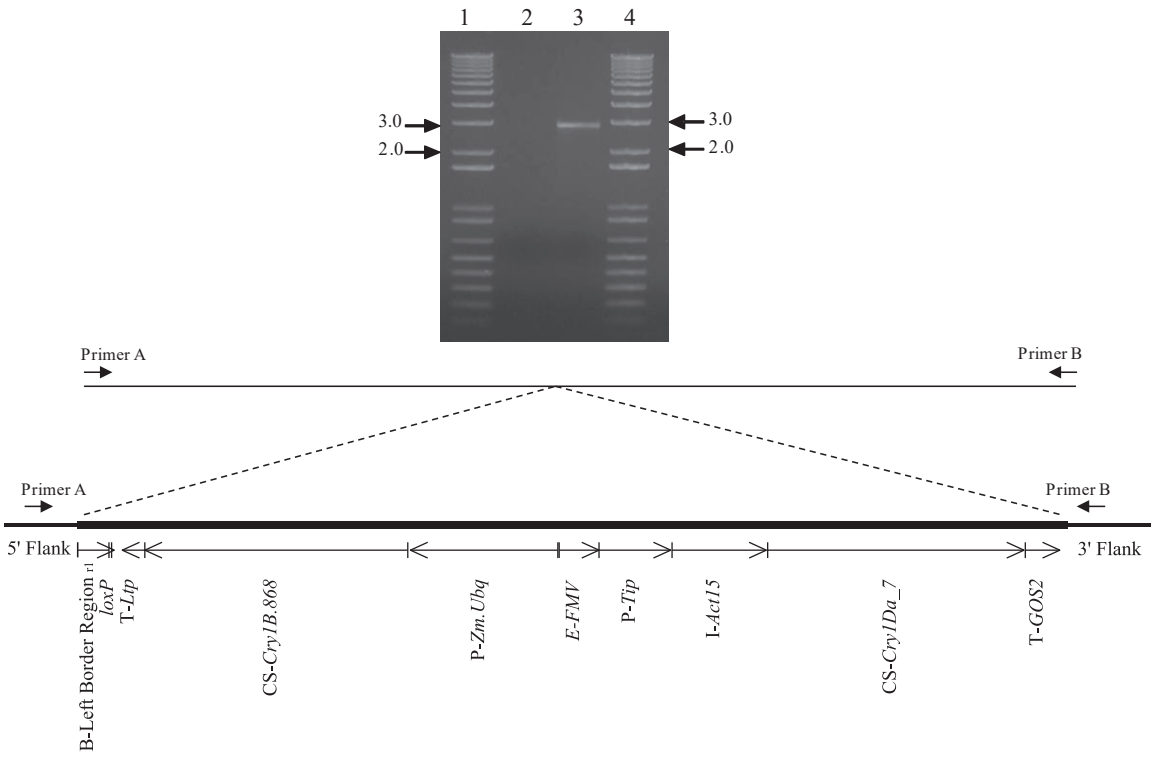

Figure IV-10. PCR Amplification of the MON 95379 Insertion Site

PCR analysis was performed to evaluate the insertion site. PCR was performed on conventional control DNA using Primer A, specific to the 5′ flanking sequence, and Primer B, specific to the 3′ flanking sequence of the insert in MON 95379. The DNA generated from the conventional control PCR was used for sequencing analysis. This illustration depicts the MON 95379 insertion site in the conventional control (upper panel) and the MON 95379 insert (lower panel). Approximately 2 µl of each of the PCR reactions was loaded on the gel. This figure is representative of the data generated in the study. Lane designations are as follows:

Lane	
1	1 Kb Plus DNA Ladder
2	No template control
3	LH244 Conventional Control
4	1 Kb Plus DNA Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb Plus DNA Ladder (Invitrogen) on the ethidium bromide stained gel.

[r1] Superscript in Left Border Regions indicate that the sequence in MON 95379 was truncated compared to the sequences in PV-ZMIR522223.

IV.E. Determination of Insert Stability over Multiple Generations of MON 95379

In order to demonstrate the stability of the T-DNA present in MON 95379 through multiple breeding generations, NGS was performed using DNA obtained from five breeding generations of MON 95379. The breeding history of MON 95379 is presented in Figure IV-4, and the specific generations tested are indicated in the figure legend. The MON 95379 (F4) generation was used for the molecular characterization analyses discussed in Sections IV.B-IV.D and shown in Figure IV-4. To assess generational stability of the T-DNA, four additional generations were evaluated by NGS as previously described in Section IV.B, and compared to the fully characterized F4 generation. The conventional controls used for the generational stability analysis included LH244, with similar background genetics to the F4 and F5 generations and represents the original transformation line; and LH244 × HCL617, a hybrid with similar background genetics to the F4F1, F5F1, F6F1 hybrids. Genomic DNA isolated from each of the selected generations of MON 95379 and conventional control was used for NGS, mapping, and subsequent junction identification (Table IV-4).

Table IV-4. Junction Sequence Classes Detected

Sample	Junction Sequence Classes Detected
MON 95379 (F4)	2
MON 95379 (F5)	2
MON 95379 (F4F1)	2
MON 95379 (F5F1)	2
MON 95379 (F6F1)	2
LH244	0
LH244 × HCL617	0

As shown by alignment to the full flank/insert sequence obtained from directed sequencing, a single conserved pair of junctions linked by contiguous known and expected DNA sequence is present in MON 95379 (F4). Two identical junctions are found in each of the breeding generations (F5, F4F1, F5F1, and F6F1), confirming the insertion of a single copy of PV-ZMIR522223 T-DNA at a single locus in the genome of MON 95379, and the consistency of these junctions in the mapping data across all generations tested demonstrates that this single locus is stably maintained throughout the MON 95379 breeding process.

These results demonstrate that the single locus of integration characterized in the F4 generation of MON 95379 is found in five breeding generations of MON 95379, confirming the stability of the insert. This comprehensive NGS and bioinformatic analysis of NGS data from multiple generations supports the conclusion that MON 95379 contains a single, stable insert T-DNA.

IV.F. Inheritance of the Genetic Insert in MON 95379

The MON 95379 T-DNA resides at a single locus within the maize genome and therefore should be inherited according to Mendelian principles of inheritance. During development of lines containing MON 95379, phenotypic and genotypic segregation data were recorded to assess the inheritance and stability of the MON 95379 T-DNA using Chi square (χ^2) analysis over several generations. The χ^2 analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 95379 breeding path for generating segregation data is described in Figure IV-11. The transformed R0 plant was self-pollinated to generate R1 seed. An individual homozygous positive plant for the MON 95379 T-DNA was identified in the R1 segregating population by Real-Time TaqMan®[3]PCR assay.

The homozygous positive R1 plant was self-pollinated to give rise to R2 seed. At the R2 generation, plants were crossed with a Cre recombinase expressing line. The Cre/*lox* technology enables the removal of DNA sequence positioned between two excision targeting sequences called *lox* sites (Hare and Chua, 2002; Zhang et al., 2003). The Cre recombinase enzyme facilitates the excision of the selectable marker cassette which was inserted during the transformation as part of the T-DNA insertion that also included the gene of interest cassettes. After excision, a single *lox* site remains in the F_1 generation. The resulting F1 progeny were self-pollinated and the F2 generation were screened for the absence of the *cre* gene (and other sequences from plasmid PV-ZMOO513642), allowing for selection of lines lacking the Cre recombinase cassette from subsequent generations and the final product. The F2 plants lacking the *cre* gene were self-pollinated to produce F3 seed. The homozygous positive F3 plant was self-pollinated to give rise to F4 seed. The homozygous positive F4 plants were crossed via traditional breeding techniques to a proprietary elite inbred parent that does not contain the *cry1B.868* and *cry1Da_7* coding sequences to produce hemizygous F4F1 seed. The hemizygous F4F1 plants were self-pollinated to produce F4F2 seed. The F4F2 generation was tested for the presence of MON 95379 T-DNA by Real Time TaqMan® PCR assay for *cry1B.868*. Hemizygous positive F4F2 plants were self-pollinated to produce F4F3 seed. The F4F3 generation was tested for the presence of the T-DNA by Real Time TaqMan® PCR assay for *cry1B.868*. Hemizygous positive F4F3 plants were self-pollinated to produce F4F4 seed. The F4F4 generation was tested for the presence of the T-DNA by Real Time TaqMan® PCR assay for *cry1B.868*.

The inheritance of the MON 95379 T-DNA was assessed in the F4F2, F4F3, and F4F4 generations. At all generations, the MON 95379 T-DNA was predicted to segregate at a 1:2:1 ratio (homozygous positive: hemizygous positive: homozygous negative) according to Mendelian inheritance principles.

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[3] TaqMan® is a registered trademark of Roche Molecular Systems, Inc.

A Pearson's chi square (χ^2) analysis was used to compare the observed segregation ratios of the MON 95379 T-DNA I coding sequence to the expected ratios.

The Chi square was calculated as:

$$\chi^2 = \sum [(\mid o - e \mid)^2 / e]$$

where o = observed frequency of the genotype or phenotype and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$).

The results of the χ^2 analysis of the segregating progeny of MON 95379 are presented in Table IV-5. The χ^2 value in the F4F2, F4F3, and F4F4 generations indicated no statistically significant difference between the observed and expected segregation ratios of MON 95379 T-DNA. These results support the conclusion that the MON 95379 T-DNA resides at a single locus within the maize genome and is inherited according to Mendelian principles of inheritance. These results are also consistent with the molecular characterization data indicating that MON 95379 contains a single intact copy of the T-DNA inserted at a single locus in the maize genome (Sections IV.B-IV.E).

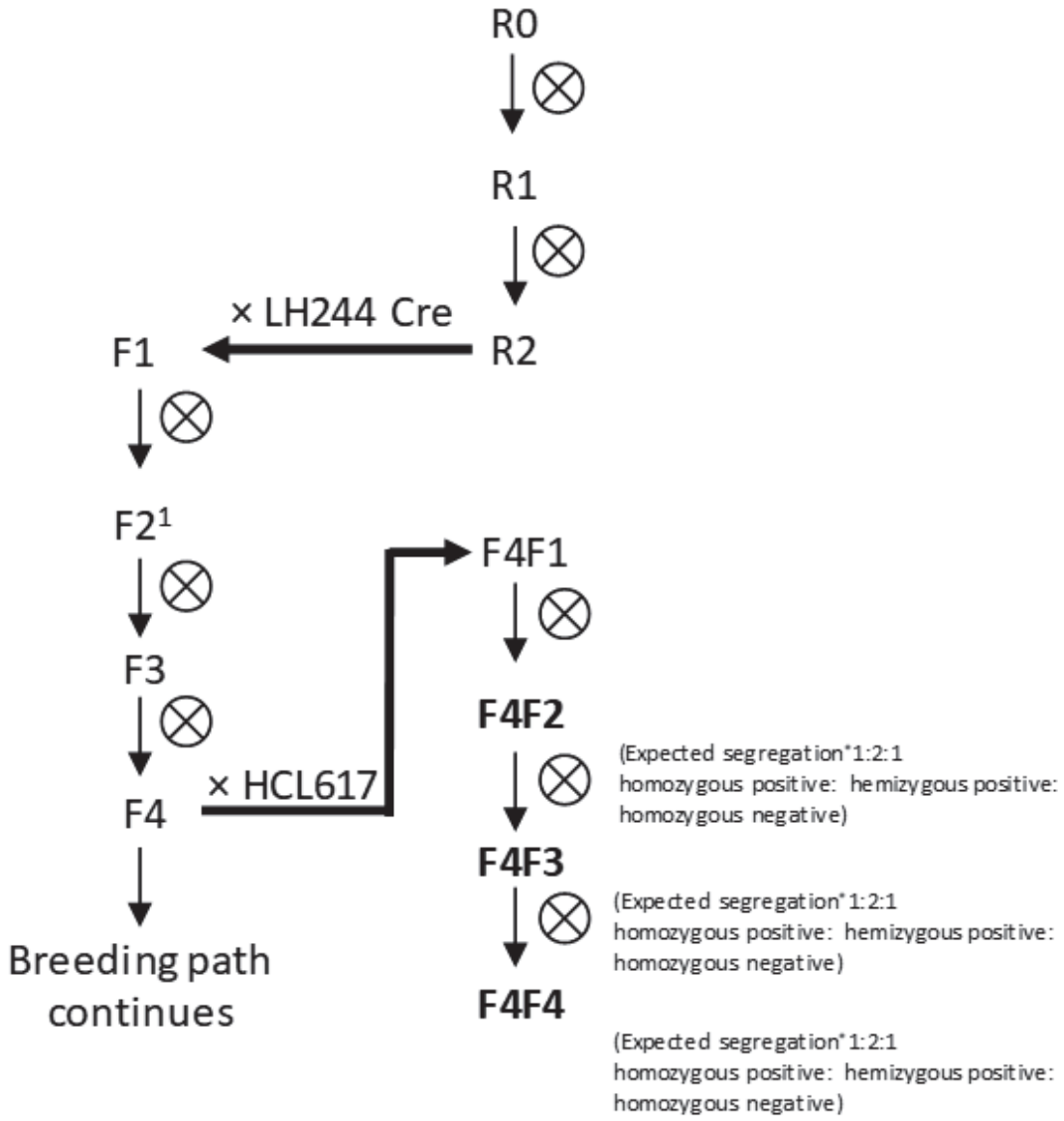

Figure IV-11. Breeding Path for Generating Segregation Data for MON 95379

*Chi-square analysis was conducted on segregation data from F4F2, F4F3, and F4F4 generations (bolded text).

⊗: Self-Pollinated

[1] The F2 generation was screened for plants lacking the *cre* gene cassette. Only those plants lacking the *cre* gene cassette were self-pollinated to create a F3 generation lacking the *cre* gene cassette.

Table IV-5. Segregation Results for MON 95379 from the F4F2, F4F3, and F4F4

					1:2:1 Segregation				
Generation	Total Plants	Observed # Plant Homozygous Positive	Observed # Plant Hemizygous Positive	Observed # Plant Homozygous Negative	Expected # Plant Homozygous Positive	Expected # Plant Hemizygous Positive	Expected # Plant Homozygous Negative	χ^2	Probability
F4F2	200	60	85	55	50	100	50	4.75	0.093
F4F3	247	62	125	60	61.75	123.5	61.75	0.07	0.966
F4F4	246	74	109	63	61.5	123	61.5	4.17	0.124

IV.G. Characterization of the Genetic Modification Summary and Conclusion

As described in this section, characterization of the genetic modification in MON 95379 was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterization demonstrate that MON 95379 contains a single copy of the intended T-DNA containing the *cry1B.868* and *cry1Da_7* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions are based on the following:

- Molecular characterization of MON 95379 by NGS demonstrated that MON 95379 contained a single intended DNA insert. These whole-genome analyses provided a comprehensive assessment of MON 95379 to determine the presence and identity of sequences derived from PV-ZMIR522223 and demonstrated that MON 95379 contains a single T-DNA insert, with no detectable backbone or *cp4 epsps* selectable marker from sequences from PV-ZMIR522223 or any sequences from PV-ZMOO513642.

- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) performed on MON 95379 was used to determine the complete sequence of the single DNA insert from PV-ZMIR522223, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the sequence and organization of the DNA is identical to the corresponding region in the PV-ZMIR522223 T-DNA. Directed sequencing also confirmed that the *cp4 epsps* selectable marker cassette which was excised, along with one *lox*P site, by Cre recombinase, is not present in the MON 95379 sequence. Furthermore, the genomic organization at the insertion site in MON 95379 was assessed by comparing the sequences flanking the T-DNA insert in MON 95379 to the sequence of the insertion site in conventional maize. This analysis determined that 160 bases were deleted at the insertion site in MON 95379 upon DNA integration.

- Generational stability analysis by NGS demonstrated that the single PV-ZMIR522223 T-DNA insert in MON 95379 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 95379.

- Segregation data confirm that the inserted T-DNA segregated according to Mendelian inheritance patterns, which corroborates the insert stability demonstrated by NGS and independently establishes the nature of the T-DNA at a single chromosomal locus.

Taken together, the characterization of the genetic modification in MON 95379 demonstrates that a single copy of the intended T-DNA was stably integrated at a single locus of the maize genome and that no PV-ZMIR522223 plasmid backbone, *cp4 epsps* selectable marker, or PV-ZMOO513642 sequences are present in MON 95379.

V. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE Cry1B.868 AND Cry1Da_7 PROTEINS PRODUCED IN MON 95379

Characterization of the introduced protein(s) in a biotechnology-derived crop is important to establishing food, feed, and environmental safety. As described in Section IV, MON 95379 contains *cry1B.868* and *cry1Da_7* expression cassettes that, when transcribed and translated, result in the expression of the Cry1B.868 and Cry1Da_7 proteins, respectively.

A multistep approach to the safety assessment of the MON 95379 Cry1B.868 and Cry1Da_7 proteins was conducted according to guidance established by the Codex Alimentarius Commission (Codex Alimentarius, 2009) and OECD, which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties. The assessment includes: 1) characterization of the physicochemical and functional properties of each expressed protein; 2) documenting the history of safe consumption of the expressed protein or its structural and functional homology to proteins that lack adverse effects on human or mammalian health; 3) examination of the similarity of each expressed protein to known allergens, toxins or other biologically active proteins known to have adverse effects on humans and other mammals; 4) evaluation of the susceptibility of each expressed protein to the digestive enzymes pepsin and pancreatin; 5) quantification of each expressed proteins' expression in plant tissues; 6) a confirmatory evaluation of potential mammalian toxicity. The safety assessment completed for MON 95379 maize supports the conclusion that exposure to the Cry1B.868 and Cry1Da_7 proteins derived from MON 95379 would not pose any risk to human or mammalian health.

V.A. Identity and Function of the Cry1B.868 and Cry1Da_7 Proteins from MON 95379

The MON 95379 Cry1B.868 and Cry1Da_7 proteins belong to the Cry three domain family of proteins that has a well-documented mode of action (Gill et al., 1992; OECD, 2007; Schnepf et al., 1998; Vachon et al., 2012). Ingestion of Cry proteins by the target insect pest exposes the protein to the alkaline conditions and proteases in the insect midgut, resulting in proteolytic cleavage of the protein's protoxin domain, thus solubilizing the parasporal inclusions and converting the protein to the active insecticidal toxin. Following activation, this protease-resistant core protein is comprised of three distinct structural domains that function in a step-wise mechanism of binding to specific membrane-embedded receptors, oligomerization at the membrane interface, insertion into the plasma membrane and pore formation leading to loss of cell integrity followed by delayed development or insect death (Bravo et al., 2007; Deist et al., 2014).

Cry1B.868 is a protein that comprises a single polypeptide of 1199 amino acids with an apparent molecular weight of approximately 127 kDa. Like other Cry proteins, Cry1B.868 is first synthesized as a prototoxin that upon exposure to the midgut of target organisms is cleaved by digestive enzymes to yield an approximately 60 kDa activated protein (Bravo et al., 2007). Cry1B.868 is a chimeric three domain protein that consists of domains I and II from Cry1Be, domain III from Cry1Ca, and the C-terminal domain from Cry1Ab (Figure V-1). Cry1A, Cry1C and Cry1B proteins are from a family of insecticidal proteins derived from various subspecies of the soil bacterium *Bacillus thuringiensis* (*Bt*) which have been used extensively in formulation for commercial biopesticides (Betz et al., 2000; Bravo et al., 2011; EFSA, 2012). Cry1B.868 was designed using a domain exchange strategy to achieve high levels of activity against target

lepidopteran insect pests. Domain exchange is a well-known mechanism in nature, resulting in diversities in Cry protein functional activity that have been described extensively in the literature (de Maagd et al., 2003; de Maagd et al., 2001). By utilizing modern molecular biological tools, a domain exchange strategy has previously been used successfully to switch the functional domains of Cry1 proteins to develop microbial biopesticides with improved specificity to lepidopteran insect pests. (Baum, 1998; Baum et al., 1999; Gao et al., 2006). Similarly, by exchanging Domain III, Cry1B.868 has been engineered to have an enhanced specificity for fall armyworm relative to Cry1Be (Wang et al., 2019). Domains I and II of Cry1B.868 are 100% identical to the respective domains of Cry1Be. Domain III of Cry1B.868 is 100% identical to domain III of Cry1Ca. The C-terminal region of Cry1B.868 is 100% identical to that of Cry1Ab.

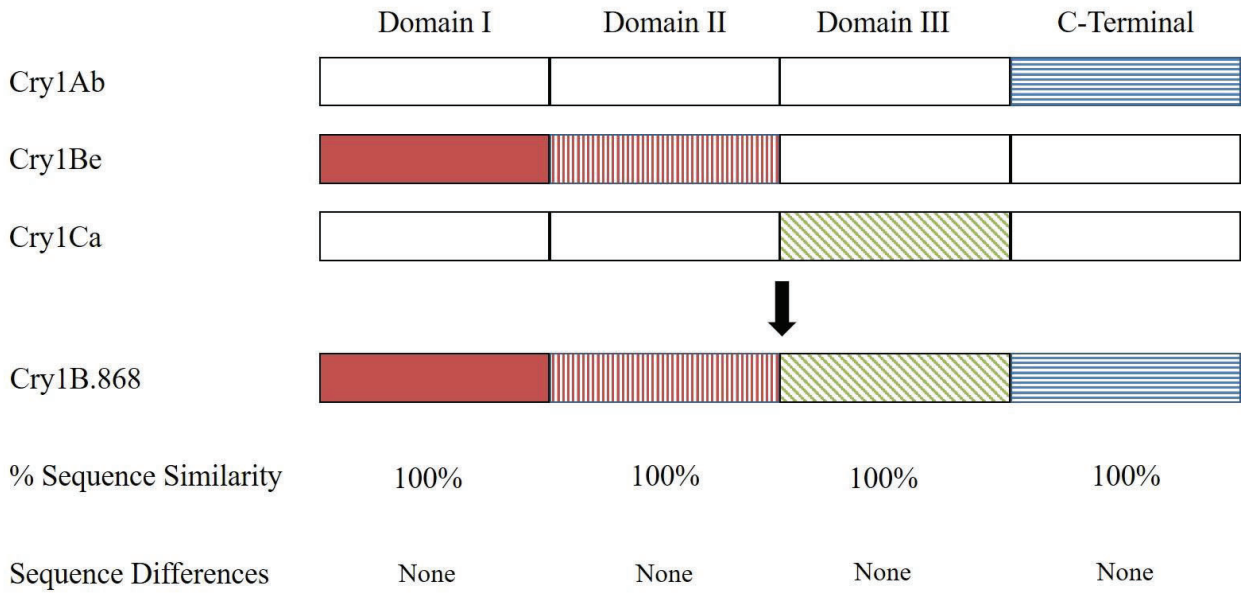

Figure V-1. Schematic Representation of the Primary Domain Architecture of the Cry1B.868 Protein

The domain architecture of the chimeric Cry1B.868 protein is schematically presented. Different shades are used to differentiate the origin of domains. For simplicity, the lengths of the domains in this illustration are not in proportion to the lengths of amino acid sequence of the respective domains.

Cry1Da_7 in MON 95379 is a protein that comprises a single polypeptide of 1166 amino acids, with an apparent molecular weight of approximately 132 kDa. Like other Cry proteins, it is first synthesized as a prototoxin that upon exposure to the midgut of target organisms is cleaved by digestive enzymes to yield an approximately 60 kDa activated protein (Bravo et al., 2007). Cry1Da_7 is a modified version of the Cry1Da protein that shares approximately 99.7% sequence identity to its parent protein (Figure V-2). Cry1Da is an insecticidal protein derived from the soil bacterium *Bt*, which has been used extensively in the formulation of commercial biopesticides (Bravo et al., 2011; EFSA, 2012). Cry1Da_7 has four distinct amino acid differences relative to Cry1Da. In Domain I, the protein has an additional alanine at position 2. This alanine was added to optimize the codon for translation of the protein *in planta*. In Domain II, there are three amino acid substitutions: serine 282 to valine, tyrosine 316 to serine, and isoleucine 368 to proline. These

amino acid substitutions result in an improvement in activity towards corn earworm relative to Cry1Da while maintaining fall army worm activity (Wang et al., 2019).

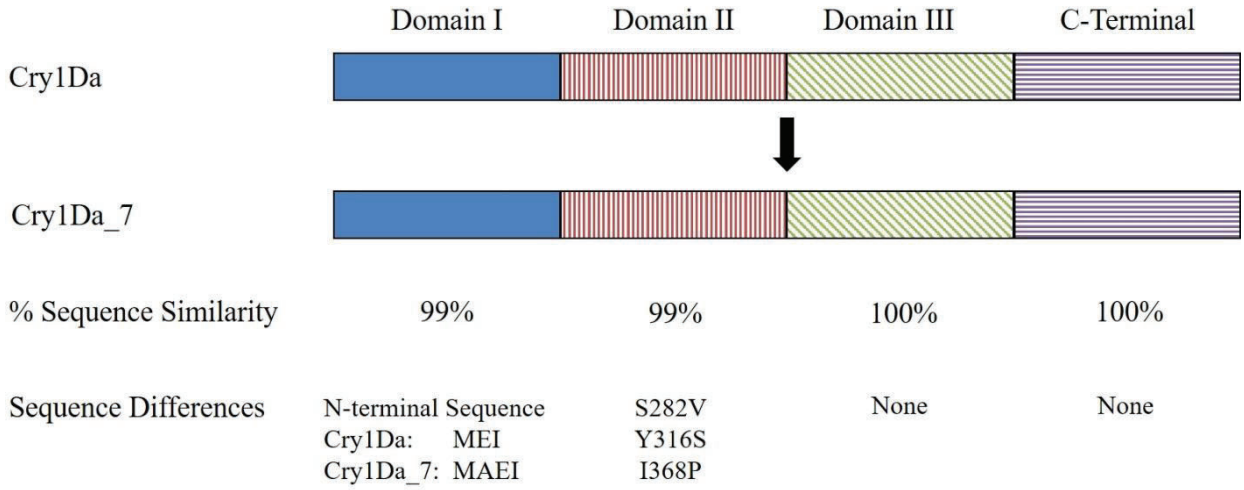

Figure V-2. Schematic Representation of the Primary Domain Architecture of the Cry1Da_7 Protein

Domain architecture of the Cry1Da_7 protein, which differs from its parent protein by only four amino acids (an alanine is added in position two in Domain I and 3 single amino acid positions in Domain II; V = valine, S = serine, Y = tyrosine, P = proline, I = isoleucine). For simplicity, the lengths of the domains in this illustration are not in proportion to the lengths of amino acid sequence of the respective domains.

V.B. Characterization and Equivalence of Cry1B.868 and Cry1Da_7 Proteins from MON 95379

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of the protein(s) produced from the inserted DNA, and confirmation of the safety of the protein(s). The expression level of Cry1B.868 and Cry1Da_7 in MON 95379 is low, and insufficient for use in the subsequent safety evaluations. Therefore, recombinant Cry1B.868 and Cry1Da_7 proteins were produced in *Bt*, using expression vectors with *cry1B.868* or *cry1Da_7* coding sequences that matched those of the *cry1B.868* or *cry1Da_7* coding sequences in MON 95379. The physicochemical and functional characteristics of the MON 95379-produced Cry1B.868 and Cry1Da_7 proteins were determined and shown to be equivalent to the *Bt*-produced Cry1B.868 and Cry1Da_7 proteins, respectively. A summary of the analytical results for the characterization and evaluation of the introduced proteins is shown below and the details of the materials, methods, and results are described in Appendix C, Appendix D and Appendix E.

The MON 95379-produced Cry1B.868 and Cry1Da_7 proteins were purified from the grain of MON 95379 and the physicochemical and functional properties were characterized using a panel of analytical tests and compared to the *Bt*-produced version of each respective protein to establish equivalence: 1) N-terminal sequence analysis of MON 95379-produced Cry1B.868 and Cry1Da_7 identified the expected N-terminal sequence; 2) liquid chromatography-mass

spectrometry (LC/MS) analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin or Asp-N digest of the MON 95379-produced Cry1B.868 and Cry1Da_7 sequences; 3) western blot analysis with antibodies specific for Cry1B.868 and Cry1Da_7 proteins demonstrated that the immunoreactive properties of each respective MON 95379-produced and *Bt*-produced protein were equivalent; 4) SDS-PAGE analysis showed that the molecular weight based on electrophoretic mobility of each respective MON 95379-produced and *Bt*-produced protein was equivalent; 5) the MON 95379-produced and *Bt*-produced MON 95379 Cry1B.868 and Cry1Da_7 proteins were determined to be non-glycosylated; and 6) insect bioassay data was used to demonstrate equivalent functional activity for each MON 95379-produced protein and its corresponding *Bt*-produced protein (See Appendix C and Appendix D).

Taken together, these data provide a detailed characterization of the MON 95379-produced Cry1B.868 and Cry1Da_7 proteins and establish their equivalence to the *Bt*-produced Cry1B.868 and Cry1Da_7 proteins, respectively. Demonstration of equivalency justifies the use of the *Bt*-produced Cry1B.868 and Cry1Da_7 proteins in studies to establish the safety of the Cry1B.868 and Cry1Da_7 proteins expressed in MON 95379.

V.C. Cry1B.868 and Cry1Da_7 Donor Organism and History of Safe Use

V.C.1. The Donor Organism has a History of Safe Use

The coding sequences for the Cry1B.868 and Cry1Da_7 proteins are derived from the bacterium *Bacillus thuringiensis*:

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: *Bacillus*

The Cry1B.868 and Cry1Da_7 proteins are derived from genetic elements that code for crystalline proteins (Cry) that are expressed as parasporal inclusions (or δ-endotoxins) in the ubiquitous gram-positive bacterium *Bacillus thuriengensis* (*Bt*) (Gill et al., 1992; Schnepf et al., 1998; Vachon et al., 2012). *Bt* isolates have a long, documented history of safe use in agriculture and safe human consumption. Since the first *Bt* isolate was registered as a pesticide in 1961, over 180 microbial *Bt* products have been registered in the United States (U.S.), with more than 120 microbial products registered in the European Union (EU) (Hammond, 2004). *Bt* microbial biopesticides have been safely and directly applied to consumed agricultural commodities including berry crops, cabbage, grapes, tomatoes, celery, lettuce, and spinach (U.S. EPA, 1998a). For certain crops, a significant percentage of the total U.S. grown crop has been treated with *Bt* crystal/spore preparations (*e.g.,* blackberries (50%), celery (46%), and cabbage (39%)) (U.S. EPA, 1998a). In Europe, residual levels of *Bt* microbials of up to 100,000 CFUs (colony forming units) were observed on fresh vegetables following application of *Bt* microbial pesticides (Frederiksen et al., 2006). Thus, the use of *Bt* microbials for pest control in agriculture, including in organic farming, provides a 50-year history of safe consumption of food crops sprayed with *Bt* microbial pesticides. Due to the

high efficacy and safety, global demand for *Bt* biopesticides, as a preferred substitution for chemical pesticides, is expected to increase 5.3% from 2018 to 2022 (Chen, 2018) and is projected to continue increasing (Damalas and Koutroubas, 2018; Seiber et al., 2014).

Several different *Bt* subspecies, including *Bt* subsp. *kurstaki* and *Bt* subsp. *aizawai*, have been subjected to toxicity testing and showed no evidence for adverse effects on human health (Baum et al., 1999; Betz et al., 2000; Federici and Siegel, 2008; Hammond, 2004; McClintock et al., 1995; Mendelsohn et al., 2003; Siegel, 2001; U.S. EPA, 1986; 2001a). These subspecies are extensively used in formulations for commercial biopesticides and express a diverse array of Cry1 proteins (e.g., Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1D and Cry1B proteins) (Betz et al., 2000). Additionally, direct toxicity assessments of *Bt* microbial biopesticide formulations containing these strains, such as Dipel®[4], Cutlass® OF[5], Crymax®[6], Xentari®[7] WG, Turex® 50 WG[8] and Thuricide®[9] have shown no evidence for adverse effects on human or mammalian health (Betz et al., 2000; Fisher and Rosner, 1959; Hadley et al., 1987; McClintock et al., 1995; U.S. EPA, 1986; 1996b; VKM, 2016).

Taken together, the long history of safe use in agriculture and the comprehensive toxicity testing of *Bt* subspecies and *Bt*-derived biopesticides provides strong support for the conclusions that the Cry1B.868 and Cry1Da_7 donor organism (*Bt*) presents no health hazard to mammals when present in food or feed or in the environment to non-target organisms beneficial to agriculture.

V.C.2. Cry Proteins have a History of Safe Use

The mode of action of Cry proteins has been extensively studied and is well-documented (Gill et al., 1992; OECD, 2007; Schnepf et al., 1998; Vachon et al., 2012). Insecticidal proteins in this family confer insect control by forming pores in the insect intestinal tract leading to developmental delay or insect death. Ingestion of Cry proteins by the target insect pest exposes the protein to the alkaline conditions and proteases in the insect midgut, which results in proteolytic cleavage of the protein's protoxin domain(s), thus solubilizing the parasporal inclusions and converting the protein to the active insecticidal toxin. Target insect specificity is mediated in part by specific insect midgut proteases that activate the Cry protein (Bravo et al., 2007; Federici and Siegel, 2008; Koch et al., 2015; Wang et al., 2018). Following activation, this protease-resistant core protein is comprised of three distinct structural domains that function in a step-wise mechanism of binding to specific membrane-embedded receptors, oligomerization at the membrane interface, insertion into the plasma membrane and pore formation (Bravo et al., 2007; Deist et al., 2014; Federici and Siegel, 2008). The specificity of Cry proteins is mediated in part by their activation by midgut

[4] Dipel is a registered trademark of Abbott, Inc.
[5] Cutlass is a registered trademark of Certis USA
[6] Crymax is a registered trademark of Ecogen, Inc.
[7] Xentari is a registered trademark of Valent USA
[8] Turex is a registered trademark of Certis USA
[9] Thuricide is a registered trademark of Certis USA

proteases and their binding to specific receptors on the brush-border membrane within the insect midgut (Deist et al., 2014; Farmer et al., 2017; Federici and Siegel, 2008; Gill et al., 1992; OECD, 2007; Schnepf et al., 1998; Vachon et al., 2012). These specific receptors are not present in humans or other mammals, nor in most of non-target insects; therefore, there is limited potential hazard related to exposure in humans and animals including the majority of non-target insects (Farmer et al., 2017; Koch et al., 2015).

Several *Bt*-derived Cry proteins that confer insect protection have been used in genetically engineered (GE) plants. Consistent with the specific mode of action of the Cry proteins, no adverse effects have been reported in mammals, birds, amphibians or reptiles after consumption of Cry proteins or Cry-protein expressing, biotechnology-derived plants (OECD, 2007; Schnepf et al., 1998). Furthermore, there are no confirmed cases of allergic reactions due to exposure or consumption of Cry proteins either through GE crops expressing Cry proteins or microbial-derived *Bt* insecticides during nearly 60 years of use (Federici and Siegel, 2008; Mendelsohn et al., 2003; Siegel, 2001; U.S. EPA, 2018). After an extensive review of the safety data on *Bt* microbial formulations the World Health Organization (WHO, 1999) concluded that:

> *"Owing to their specific mode of action, Bt products are unlikely to pose any hazard to humans or other vertebrates or to the great majority of non-target vertebrates provided they are free from non-Bt microorganisms and biologically active products other than ICPs (insect control proteins)."*

The US EPA has established separate tolerance exemptions for various Cry proteins (e.g., Cry1Ab, Cry1Ac, Cry1A.105, Cry1F, Cry2Ab2 and Cry3A, Cry3Bb1, Cry34/35, Cry51Aa2) expressed in GE crops (U.S. EPA, 1995a; b; 1996a; 2004a; b; c; 2005b; a; 2006a; b; 2018). The USDA has deregulated GE crops containing these Cry proteins. Similarly, many global regulatory agencies have reviewed and affirmed the safety of numerous Cry proteins (ILSI-CERA, 2010; 2011; 2013b; a; c; 2014; Koch et al., 2015). Due to a lack of adverse toxic and allergenic effects of a wide array of *Bt* spray formulations, crops derived from biotechnology and Cry proteins in food or feed, there is a global consensus of reasonable certainty of no harm when Cry proteins are consumed by mammals and other terrestrial vertebrates.

V.C.3. Cry1B.868 Protein has a History of Safe Use

The Cry1B.868 protein in MON 95379 is a chimeric protein containing domains derived from wild-type Cry1 proteins of the Cry1A, Cry1B, and Cry1C subclasses (Figure V-1, Panel A) which are expressed natively in *Bt* and are therefore present in biopesticide formulations (e.g., Cry1A and Cry1C proteins in Xentari® and CryMax®) (Akhurst et al., 2003; Sanahuja et al., 2011). The Cry1B.868 protein provides enhanced specific activity towards fall armyworm (FAW) relative to the parental Cry1Be protein, primarily due to the substitution of Domain III with the corresponding domain from Cry1C that is recognized as having high *Spodoptera spp.* activity. The genetic recombination of sequences encoding domains from different Cry proteins is a naturally occurring biological process (Bravo et al., 2013; de Maagd et al., 2001). Domain recombination, or "domain-swapping", can also generate chimeric Cry proteins that possess increased insecticidal efficacy and/or expanded spectrum of activity while maintaining a common mode of action (Wang et al., 2018). For example, the chimeric Cry1A.105 protein, expressed in the current commercialized GE

maize products containing MON 89034 (FDA BNF No. 107, 2007[10]) including VT Double PRO® and Genuity SmartStax®, is an engineered chimeric Cry protein comprised of domains from Cry1Ab, Cry1Ac (from *Bt* subsp. *kurstaki*) and Cry1F (from *Bt* subsp. *aizawai*). A detailed comparison of the physicochemical, structural and functional properties of Cry1A.105 to its donor proteins, along with a review of the acute oral toxicity assessments of each donor protein and the chimeric protein, supports that the history of safe use of donor protein domains are applicable to a conclusion of safety for chimeric proteins (Wang et al., 2018).

The donor proteins from which the Cry1B.868 protein domains are derived have a strong safety profile. These proteins or proteins from the same subclass of Cry1 proteins, have been directly assessed or are present in the *Bt* microbial products that have been directly assessed for mammalian toxicity. The results from these toxicity assessments indicated that there was no evidence for toxicity at high doses relative to anticipated human and mammalian exposures (Betz et al., 2000; James, 2006; Koch et al., 2015; McClintock et al., 1995; Pellegrino et al., 2018; U.S. EPA, 1986; 2007). In addition, it is well-documented that Cry1 proteins become denatured and lose their functional activity when exposed to temperatures commonly used during food/feed processing and are rapidly degraded upon exposure to gastrointestinal proteases (Hammond and Jez, 2011; Wang et al., 2018). Thus, processing and digestion of foods and/or feeds further diminish exposure of functional, intact Cry1 proteins.

V.C.4. Cry1Da_7 Protein has a History of Safe Use

To improve the insecticidal activity of Cry1Da, Cry1Da_7 was designed with minor amino acid changes. Improved insecticidal activity due to minor amino acid differences has been previously reported (BenFarhat-Touzri et al., 2019; Gowda et al., 2016). The Cry1Da_7 protein in MON 95379 shares a high (99.7%) level of amino acid sequence identity to the Cry1Da protein. Proteins from the Cry1Da subclass are co-expressed natively along with Cry1Ab and Cry1Ca in *Bt* subsp. *aizawai* (Chang et al., 2001), which is the active ingredient in several commercial biopesticide formulations (notably, Xentari® and Turex®) (Chang et al., 2001; Sanahuja et al., 2011; VKM, 2016). As shown schematically in Figure V-2, the Cry1Da_7 protein differs from the wild-type Cry1Da protein by only four amino acids: an additional alanine is present at amino acid position two of the protein and three amino acid substitutions are present in Domain II (serine 282 to valine, tyrosine 316 to serine, isoleucine 368 to proline). These single amino acid variations resulted in a 50-fold improvement in activity towards *Helicoverpa zea* (corn earworm, CEW) while not reducing activity towards *Spodoptera frugiperda* (fall armyworm, FAW).

A recent assessment comparing the weight of evidence for the protein safety of two variants of Cry51Aa2.834, another Cry protein, demonstrated that minor amino acid sequence modifications resulted in an increase in insecticidal activity but did not alter the protein's hazard to mammals

[10] https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=Biocon&id=MON-89034-3

(Farmer et al., 2017; Gowda et al., 2016; Koch et al., 2015). Given the highly-specific mode of action of Cry proteins these results are not surprising, as the specific receptors for Cry proteins are not found in taxa outside of insects (Gill et al., 1992; OECD, 2007; Schnepf et al., 1998; Vachon et al., 2012).

V.D. Cry1B.868 and Cry1Da_7 Proteins in MON 95379 are Not Homologous to Known Allergens or Toxins

Bioinformatics analyses were performed to assess the potential for allergenicity or toxicity of the Cry1B.868 and Cry1Da_7 proteins. The allergen, gliadin, and glutenin protein sequence database (AD_2019) was obtained as the "COMprehensive Protein Allergen REsource" (COMPARE) database from the Health and Environmental Sciences Institute (HESI). The AD_2019 curated database contains 2,081 sequences.

The allergen bioinformatic results demonstrated there were no biologically relevant sequence similarities to allergens when the Cry1B.868 and Cry1Da_7 protein sequences were used as a query for a FASTA search of the publicly available allergen (AD_2019) database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the Cry1B.868 and Cry1Da_7 protein sequences and proteins in the allergen database. These data show that the Cry1B.868 and Cry1Da_7 protein sequences lack both structurally and immunologically relevant similarities to known allergens, gliadins, and glutenins.

FASTA bioinformatic alignment searches using the Cry1B.868 and Cry1Da_7 amino acid sequences were performed with a toxin database to identify possible homology with proteins that may be harmful to human and animal health. The toxin database, TOX_2019, is a subset of sequences derived from the PRT_2019 database (which is an all protein GenBank release downloaded from NCBI[11]), that was selected using a keyword search and filtered to remove likely non-toxin proteins. The TOX_2019 database contains 34,642 sequences and has been curated to remove *Bt* insecticidal Cry proteins. The results of the bioinformatic analyses demonstrated that no relevant similarity exists between the Cry1B.868 and Cry1Da_7 proteins and any sequence in the TOX_2019 database.

These analyses demonstrated that the Cry1B.868 and Cry1Da_7 proteins do not share amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins which could have adverse effects on human or animal health.

[11] https://www.ncbi.nlm.nih.gov/search/all/?term=GenBank

V.E. Cry1B.868 and Cry1Da_7 Proteins in MON 95379 are Susceptible to Degradation in *in vitro* Digestion Assays

One characteristic of several food allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood et al., 1996; Moreno et al., 2005; Vassilopoulou et al., 2006; Vieths et al., 1999). The enzymatic degradation of an ingested protein by exposure to gastric pepsin and/or intestinal pancreatic proteases (e.g., pancreatin) makes it highly unlikely that either the intact protein or protein fragment(s) will reach the absorptive epithelial cells of the small intestine where antigen processing cells reside (Moreno et al., 2005). The susceptibility of the MON 95379-produced Cry1B.868 and Cry1Da_7 to degradation by pepsin and pancreatin was assessed using the *Bt*-produced Cry1B.868 and Cry1Da_7 proteins, which were shown to be equivalent to the MON 95379-produced Cry1B.868 and Cry1Da_7 (see Appendix F). The results indicated that the full-length, *Bt*-produced Cry1B.868 and Cry1Da_7 proteins are readily degraded by pepsin and pancreatin. Peptide fragments of ~4 kDa that were resistant (i.e., present over the course of the 60 min digestive reaction) to pepsin degradation were observed for both proteins. To better understand the fate of the detected peptide fragments, sequential degradation of the Cry1B.868 and Cry1Da_7 proteins in pepsin followed by pancreatin was conducted. The results indicated that the fragments are transient as they were readily degraded by sequential digestion. As expected, Cry1B.868 and Cry1Da_7 subjected to pancreatin were each processed to a trypsin-resistant core that was stable throughout the pancreatin digestion period. These results are consistent with observations for these and other Cry proteins subjected to pancreatin digestion. Thus, evidence supports the conclusion that gastrointestinal digestion is sufficient to degrade the intact Cry1B.868 and Cry1Da_7 proteins and any fragments thereof making it highly unlikely that intact or large peptide fragments of Cry1B.868 and Cry1Da_7 proteins would be absorbed in the small intestine and have the potential to impact human or mammalian health.

V.F. Expression Levels of Cry1B.868 and Cry1Da_7 Proteins in MON 95379

Cry1B.868 and Cry1Da_7 protein levels determined in MON 95379 are used to assess potential exposure to the introduced proteins via food or feed ingestion or potential environmental exposure. Section VII.C provides specific results of Tier I toxicity testing on NTOs with Cry1B.868 and Cry1Da_7 as well as the MON 95379 environmental risk assessment. To support environmental exposure scenarios, the most appropriate tissues to evaluate Cry1B.868 and Cry1Da_7 protein levels are leaf [over season leaf 1 (OSL1) and over season leaf 4 (OSL4)], root [over season root 1 (OSR1) and forage root] silk, pollen, forage and grain tissue samples. Levels of the introduced proteins were determined in forage and grain tissue to evaluate food and feed exposure of each protein to humans and mammals. Cry1B.868 and Cry1Da_7 protein levels in various tissues of MON 95379 relevant to the characterization and environmental assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA) assay as part of the characterization of MON 95379 (Appendix E).

Tissues of MON 95379 were collected from four replicate plots planted in a randomized complete block design during the 2018 growing season from five field sites in the United States that were representative of maize-producing regions. OSL1, OSR1, OSL4, silk, pollen, forage, forage root and grain tissue samples were collected from each replicated plot at all field sites.

V.F.1. Expression Levels of Cry1B.868 Protein

Cry1B.868 protein levels were determined in leaf, root, silk, pollen, forage and grain tissues. The results obtained from the ELISA are summarized in Table V-1 and the details of the materials and methods are described in Appendix E. The expression of Cry1B.868 in MON 95379 maize was determined and reported on a dry weight basis. Dry weight (dw) values were converted to a µg/g fresh weight (fw) using a moisture conversion factor (Appendix E). The mean Cry1B.868 protein levels in MON 95379 across all sites was highest in OSL1 at 630 µg/g dw and lowest in forage root at 22 µg/g dw.

Table V-1. Summary of Cry1B.868 Protein Levels in Maize Tissues Collected from MON 95379 Produced in United States Field Trials in 2018

Tissue Type	Development Stage[1]	Mean (SE) Range (µg/g fw)[2]	Mean (SE) Range (µg/g dw)[2]	LOD/LOQ (µg/g dw)[3]
OSL1	V2-V4	94 (3.3) 46 – 110	630 (22) 310 - 760	0.348/0.625
OSR1	V2-V4	12 (0.90) 7.3 - 23	110 (8.2) 67 - 210	0.254/0.625
OSL4	VT-R1	49 (2.6) 28 - 69	210 (11) 120 - 300	0.348/0.625
Silk	R1	6.7 (0.23) 5.3 - 8.6	67 (2.3) 53 - 86	0.380/0.625
Pollen	R1	52 (1.2) 42 - 63	91 (2.1) 73 - 110	0.323/0.625
Forage	R5	32 (2.5) 15 - 52	110 (8.2) 50 - 170	0.455/0.625
Forage Root	R5	4.3 (0.67) 1.3 - 14	22 (3.5) 6.7 - 76	0.254/0.625
Grain	R6	23 (3.1) 6.9 - 68	26 (3.5) 7.8 - 77	0.420/0.625

[1] The crop development stage at which each tissue was collected (Abendroth et al., 2011).
[2] Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fw) and dry weight basis (dw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n = 20).
[3] LOQ = limit of quantitation, LOD = limit of detection.

V.F.2. Expression Levels of Cry1Da_7 Protein

Cry1Da_7 protein levels were determined in leaf, root, silk, pollen, forage and grain tissues. The results obtained from the ELISA are summarized in Table V-2 and the details of the materials and methods are described in Appendix E. The expression of Cry1Da_7 in MON 95379 maize was determined and reported on a dry weight basis. Dry weight (dw) values were converted to a µg/g fresh weight (fw) using a moisture conversion factor (Appendix E). The mean Cry1Da_7 protein levels in MON 95379 across all sites was highest in OSL1 at 92 µg/g dw and lowest in pollen at less than the limit of quantification (LOQ) µg/g dw.

Table V-2. Summary of Cry1Da_7 Protein Levels in Maize Tissues Collected from MON 95379 Produced in United States Field Trials in 2018

Tissue Type	Development Stage[1]	Mean (SE) Range (µg/g fw)[2]	Mean (SE) Range (µg/g dw)[2]	LOD/LOQ (µg/g dw)[3]
OSL1	V2-V4	14 (0.78) 8.4 - 21	92 (5.2) 56 - 140	0.061/0.125
OSR1	V2-V4	4.7 (0.34) 2.8 - 7.9	43 (3.1) 26 - 72	0.065/0.125
OSL4	VT-R1	10 (0.72) 6.6 - 16	44 (3.1) 29 - 68	0.061/0.125
Silk	R1	0.91 (0.045) 0.58 – 1.2	9.1 (0.45) 5.8 - 12	0.080/0.125
Pollen	R1	<LOQ	<LOQ	0.065/0.125
Forage	R5	7.8 (0.63) 3.8 - 15	26 (2.1) 13 - 50	0.078/0.125
Forage Root	R5	1.8 (0.12) 0.99 – 2.9	9.6 (0.64) 5.2 - 15	0.065/0.125
Grain	R6	0.22 (0.028) 0.11 – 0.56	0.25 (0.032) 0.13 - 0.64	0.037/0.050

[1] The crop development stage at which each tissue was collected (Abendroth et al., 2011).
[2] Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fw) and dry weight basis (dw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue with values above the LOQ across all sites (n = 20).
[3] LOQ = limit of quantitation, LOD = limit of detection.

V.G. Cry1B.868 and Cry1Da_7 Proteins are Not Acutely Toxic

Acute oral toxicology studies with Cry1B.868 and Cry1Da_7 proteins were conducted to provide further confirmation of the mammalian safety of these proteins. There was no evidence of acute toxicity in mice when dosed orally at a limit dose of 5,000 mg/kg body weight (bw) with either the Cry1B.868 or the Cry1Da_7 protein, which far exceeds any exposure scenario (Appendix J, Section J.15 and J.27, respectively). Based on an absence of toxicity in the acute oral toxicity studies with the Cry1B.868 and Cry1Da_7 proteins and the relatively low dietary exposure to the protein (i.e., low expression levels of the Cry1B.868 and Cry1Da_7 proteins present in MON 95379 grain and forage (Section V.F.1; Table V-1 and Table V-2) the risk to humans and mammals following dietary exposure to Cry1B.868 and Cry1Da_7 proteins from consumption of food or feeds derived from MON 95379 is considered to be negligible.

V.H. Assessment of Potential Allergenicity of the Cry1B.868 and Cry1Da_7 Proteins

The allergenic potential of an introduced protein is assessed by comparing the biochemical characteristics of the introduced protein to biochemical characteristics of known allergens (Codex Alimentarius, 2009). Using a weight of evidence approach, a protein is not likely to be associated with allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein represents a small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence; and 4) the protein shows susceptibility to pepsin and pancreatin treatments.

The Cry1B.868 and Cry1Da_7 proteins have been assessed for potential allergenicity according to these safety assessment guidelines.

1) Cry1B.868 and Cry1Da_7 proteins originate from *Bt*, an organism that has not been reported to be a source of known allergens.

2) Cry1B.868 and Cry1Da_7 proteins represent a small portion of the total protein in the grain that could be consumed from MON 95379 maize due to very low expression in grain.

3) Bioinformatics analyses demonstrated that the Cry1B.868 and Cry1Da_7 proteins do not share amino acid sequence similarities with known allergens and, therefore, are highly unlikely to contain immunologically cross-reactive allergenic epitopes.

4) Finally, *in vitro* experiments conducted with the Cry1B.868 and Cry1Da_7 proteins demonstrated that the proteins are rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) under physiological conditions.

Taken together, these data support the conclusion that the Cry1B.868 and Cry1Da_7 proteins produced in MON 95379 do not pose a significant allergenic risk to humans or mammals.

V.I. Mammalian Exposure to the Cry1B.868 and Cry1Da_7 Proteins in MON 95379

The ubiquitous donor organism *Bt* and Cry protein family have a long, documented history of safe use in food and feed. The MON 95379 Cry1B.868 and Cry1Da_7 proteins lack structural similarity to known allergens or mammalian toxins. Cry1B.868 and Cry1Da_7 are susceptible to the digestive enzymes pepsin and pancreatin and because of the relatively low expression levels of Cry1B.868 and Cry1Da_7 proteins present in MON 95379 grain, there is relatively low dietary exposure to the proteins. In addition, there was an absence of toxicity for the Cry1B.868 and Cry1Da_7 proteins in a confirmatory acute oral toxicity study. Based on these factors, it is unlikely that exposure to the Cry1B.868 and Cry1Da_7 proteins in food and feed products derived from MON 95379 pose a risk to human and other mammals.

V.J. Cry1B.868 and Cry1Da_7 Proteins Characterization and Safety Conclusion

The Cry1B.868 and Cry1Da_7 proteins are members of the three-domain Cry family of insecticidal proteins which have a well-documented history of safe use and mode of action. The physicochemical and functional characteristics of the Cry1B.868 and Cry1Da_7 proteins from MON 95379 were determined and shown to be equivalent to those of their *Bt*-produced counterparts. This equivalence justifies the use of the *Bt*-produced Cry1B.868 and Cry1Da_7 proteins as test substances in the protein safety studies, including acute oral toxicity and digestibility studies. Expression studies using immunoassay demonstrated that Cry1B.868 and Cry1Da_7 were expressed at low levels in grain, forage and other tissues evaluated. The Cry1B.868 and Cry1Da_7 proteins lack structural similarity to allergens, toxins or other proteins known to have adverse effects on mammals. In addition, the donor organism for the Cry1B.868 and Cry1Da_7 coding sequences, *B. thuringiensis*, is ubiquitous in the environment and not known for human or mammalian pathogenicity or allergenicity. The Cry1B.868 and Cry1Da_7 proteins are rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) and demonstrated no acute oral toxicity in mice at the highest level tested, which far exceeds any exposure scenario. Taken together, these findings support the conclusion that the consumption of the Cry1B.868 and Cry1Da_7 proteins in food and feed derived from MON 95379 or its progeny poses no risk to human and mammalian health.

The protein safety data presented herein support the overall conclusion that food and feed products containing MON 95379 or derived from MON 95379 are as safe for human and mammalian consumption as maize currently on the market and expression of the Cry1B.868 and Cry1Da_7 proteins do not impact the plant pest risk of MON 95379 maize.

VI. COMPOSITIONAL ASSESSMENT OF MON 95379

For MON 95379, the introduced Cry1B.868 and Cry1Da_7 proteins confer protection from lepidopteran pests and lack catalytic activity that is intended or expected to affect the plant's metabolism. Given the nature of these introduced traits and the overall lack of meaningful unintended compositional characteristics observed for biotechnology-derived products characterized to date, compositional changes that would affect the levels of nutritional components in MON 95379 maize were not expected.

Food and feed safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius, 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Maize is not known to have any endogenous toxicants or anti-nutrients associated with overall plant pest potential (OECD, 2002a). For maize, assessments are performed using the general principles outlined in the OECD consensus document for maize composition (OECD, 2002a).

A review of compositional assessments conducted according to OECD guidelines, that encompassed a total of seven biotechnology-derived crop varieties, nine countries, and eleven growing seasons, concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition. Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan et al., 2010; Zhou et al., 2011). Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan et al., 2010; Harrigan et al., 2009; Ridley et al., 2011; Zhou et al., 2011).

Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2002b). OECD consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients. These quantitative measurements effectively discern any compositional changes that imply potential nutritional or safety (e.g., anti-nutritional) concerns. Levels of the components in grain and/or other raw agricultural commodities of the biotechnology-derived crop product are compared to: 1) corresponding levels in a conventional comparator, a genetically similar conventional line, grown concurrently under similar field conditions, and 2) natural ranges reported in ILSI Crop Composition Database and from data published in the scientific literature and from data published in the scientific literature. The comparison to data published in the literature places any potential differences between the assessed crop and its comparator in the context of the well-documented variation in the concentrations of crop nutrients and anti-nutrients.

This section provides a summary of the analyses conducted to evaluate the levels of key nutrients and anti-nutrients in grain and forage of MON 95379 compared to that of a conventional control maize (LH244 x HCL617) grown and harvested under similar conditions. The production of materials for compositional analyses used a sufficient variety of field trial sites, robust experimental design (randomized complete block design with four blocks), and sensitive analytical

methods that allow accurate assessments of compositional characteristics over a range of environmental conditions typical for corn production. See Appendix I for details on composition methods.

VI.A. Compositional Equivalence of MON 95379 Grain and Forage to Conventional Maize

Grain and forage samples were collected from MON 95379 and a conventional control at five sites grown in the United States during the 2018 season. The field sites were planted in a randomized complete block design with four blocks per site. MON 95379 and the conventional control were grown under agronomic field conditions typical for the different growing regions.

Compositional analyses of grain and forage samples are reported for the key components listed in the maize OECD consensus document (OECD, 2002a). Harvested grain samples were assessed for moisture and levels of key nutrients, including proximates (protein, total fat and ash; Table VI-1), essential amino acids (10 components; Table VI-2), the essential fatty acid linoleic acid (Table VI-1), carbohydrates by calculation and fiber (acid detergent fiber (ADF); and neutral detergent fiber (NDF) Table VI-1). Grain samples were also assessed for levels of anti-nutrients (phytic acid and raffinose; Table VI-3). Harvested forage samples were assessed for moisture and key levels of nutrients, including proximates (protein, total fat and ash; Table VI-4), carbohydrates by calculation and fiber (ADF and NDF; Table VI-4). In all, 27 different components were analyzed. Moisture values for grain and forage were measured to enable the conversion of components from fresh to dry weight but were not statistically analyzed. Therefore, 25 components were statistically analyzed.

The statistical comparison of MON 95379 and the conventional control was based on compositional data that was combined across all field sites. Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). A statistically significant difference between MON 95379 and the conventional control does not necessarily imply biological relevance from a food and feed perspective. Therefore, statistically significant differences observed between MON 95379 and the conventional control were evaluated further to determine whether the detected difference indicated a biologically relevant compositional change or supported a conclusion of compositional equivalence, as follows:

Step 1 – Determination of the Magnitude of Difference between MON 95379 and Conventional Control Means

The difference in mean values between MON 95379 and the conventional control was determined for use in subsequent steps.

Step 2 – Assessment of the Difference in the Context of Natural Variation within the Conventional Control

The relative impact of MON 95379 was evaluated in the context of variation within the conventional control germplasm grown across multiple sites. This step assessed the mean difference between MON 95379 and the conventional control in the context of the conventional control range value (maximum value minus the minimum value). When a mean difference is less than the variability seen due to natural environmental variation within the conventional control, the difference is typically not a food or feed safety concern (Venkatesh et al., 2014).

Step 3 – Assessment of the Difference in the Context of Natural Variation Due to Multiple Factors

The relative impact of MON 95379 on composition was also evaluated in the context of sources of natural variation such as environmental and germplasm influences. This assessment determined whether the component mean value of MON 95379 was within the natural variability defined by the literature values and/or the ILSI Crop Composition Database (ILSI-CCDB, Table VI-5) values. This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition between MON 95379 and the conventional control.

These evaluations of natural variation are important as crop composition is known to be greatly influenced by environment and variety (Harrigan et al., 2010). Although used in the comparative assessment process, detection of statistically significant differences between MON 95379 and the conventional control mean values does not imply a meaningful contribution by MON 95379 to compositional variability. Only if the impact of MON 95379 on levels of components was large relative to natural variation inherent to conventional maize would further assessments be required to establish whether the change in composition would have an impact from a food and feed safety and nutritional perspective. The steps reviewed in this assessment therefore describe the process for determining whether the differences between MON 95379 and the conventional control are meaningful from a food and feed safety perspective or whether they support a conclusion of compositional equivalence.

VI.B. Compositional Assessment of MON 95379 Conclusion

Compositional analysis was conducted on grain and forage of MON 95379 and the conventional control grown at five sites in the United States during the 2018 field season. Of the 25 components statistically assessed, 17 showed no statistically significant differences ($p<0.05$) between MON 95379 and the conventional control. A total of 8 components in grain (protein, carbohydrates, isoleucine, leucine, methionine, phenylalanine, threonine, and valine) showed a statistically significant difference ($p<0.05$) between MON 95379 and the conventional control. For these components, the mean difference in component values between MON 95379 and the conventional control was less than the range of the conventional control values (Table VI-1 and Table VI-2). The MON 95379 mean component values were within the range of values observed in the literature and/or the ILSI-CCDB (Table VI-5).

These results support the overall conclusion that MON 95379 maize was not a major contributor to variation in component levels in grain or forage and confirmed the compositional equivalence of MON 95379 to the conventional control in levels of key nutrients and anti-nutrients in grain and forage. The statistically significant differences observed were not compositionally meaningful from a food and feed safety perspective. In addition, these results support the overall food and feed safety of MON 95379.

Table VI-1. Summary of Maize Grain Proximates, Linoleic Acid, Carbohydrates by Calculation and Fiber for MON 95379 and the Conventional Control

				Difference (MON 95379 minus Control)	
Component	MON 95379 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Mean (S.E.)	p-Value
Protein (% dw)[3]	9.61 (0.56) 7.97 - 12.47	9.18 (0.56) 7.54 - 11.58	4.04	0.43 (0.16)	0.012
Total fat (% dw)	3.86 (0.038) 3.69 - 4.08	3.80 (0.038) 3.59 - 4.06	0.47	0.059 (0.039)	0.204
Linoleic acid (% Total FA)[4]	55.24 (0.37) 53.67 - 56.93	54.99 (0.37) 52.94 - 56.19	3.26	0.25 (0.16)	0.126
Ash (% dw)	1.29 (0.024) 1.14 - 1.42	1.28 (0.024) 1.12 - 1.41	0.29	0.012 (0.022)	0.607
Carbohydrates by calculation (% dw)	85.23 (0.61) 82.24 - 87.10	85.73 (0.61) 83.22 - 87.53	4.31	-0.50 (0.16)	0.005
ADF (% dw)	3.65 (0.098) 2.92 - 4.55	3.51 (0.098) 2.71 - 4.17	1.45	0.14 (0.12)	0.317
NDF (% dw)[3]	9.26 (0.15) 7.54 - 10.28	9.26 (0.15) 8.67 - 10.52	1.86	-0.0022 (0.22)	0.992

[1]Mean (S.E.) = least-square mean (standard error) obtained from the linear mixed model analysis
[2]Maximum value minus minimum value for the control maize hybrid
[3]dw = dry weight
[4]FA = Fatty Acid

Table VI-2. Summary of Maize Grain Essential Amino Acids for MON 95379 and Conventional Control

Component (% dw)[1]	MON 95379 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Difference (MON 95379 minus Control) Mean (S.E.)	p-Value
Arginine	0.48 (0.019) 0.40 - 0.56	0.46 (0.019) 0.40 - 0.53	0.13	0.016 (0.0082)	0.062
Histidine	0.25 (0.013) 0.20 - 0.32	0.25 (0.013) 0.21 - 0.31	0.11	0.0063 (0.0048)	0.204
Isoleucine	0.34 (0.023) 0.28 - 0.45	0.33 (0.023) 0.27 - 0.43	0.16	0.015 (0.0059)	0.019
Leucine	1.24 (0.10) 0.93 - 1.74	1.17 (0.10) 0.91 - 1.62	0.71	0.070 (0.026)	0.012
Lysine	0.26 (0.0062) 0.21 - 0.30	0.26 (0.0062) 0.22 - 0.31	0.087	0.0011 (0.0067)	0.874
Methionine	0.22 (0.013) 0.18 - 0.29	0.21 (0.013) 0.17 - 0.27	0.098	0.012 (0.0042)	0.008

Table VI-2. Summary of Maize Grain Essential Amino Acids for MON 95379 and Conventional Control (continued)

Component (% dw)[1]	MON 95379 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Difference (MON 95379 minus Control) Mean (S.E.)	Difference (MON 95379 minus Control) p-Value
Phenylalanine	0.49 (0.035) 0.40 - 0.67	0.47 (0.035) 0.38 - 0.63	0.25	0.023 (0.0093)	0.025
Threonine	0.34 (0.018) 0.30 - 0.44	0.33 (0.018) 0.28 - 0.41	0.12	0.011 (0.0052)	0.048
Tryptophan	0.077 (0.0036) 0.065 - 0.097	0.074 (0.0036) 0.066 - 0.090	0.025	0.0027 (0.0013)	0.106
Valine	0.45 (0.024) 0.39 - 0.57	0.43 (0.024) 0.36 - 0.53	0.17	0.016 (0.0076)	0.038

[1]dw = dry weight
[2]Mean (S.E.) = least-square mean (standard error) obtained from the linear mixed model analysis
[3]Maximum value minus minimum value for the control maize hybrid

Table VI-3. Summary of Maize Grain Anti-Nutrients for MON 95379 and Conventional Control

Component (% dw)[1]	MON 95379 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Difference (MON 95379 minus Control) Mean (S.E.)	Difference (MON 95379 minus Control) p-Value
Phytic acid	0.74 (0.034) 0.46 - 0.91	0.70 (0.034) 0.49 - 0.92	0.43	0.045 (0.045)	0.351
Raffinose	0.21 (0.014) 0.17 - 0.27	0.19 (0.014) 0.16 - 0.26	0.11	0.011 (0.0053)	0.105

[1]dw = dry weight
[2] Mean (S.E.) = least-square mean (standard error) obtained from the linear mixed model analysis
[3]Maximum value minus minimum value for the control maize hybrid

Table VI-4. Summary of Maize Forage Proximates, Carbohydrates by Calculation and Fiber for MON 95379 and Conventional Control

Component (% dw)[1]	MON 95379 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Difference (MON 95379 minus Control) Mean (S.E.)	Difference (MON 95379 minus Control) p-Value
Protein	6.95 (0.38) 5.52 - 8.69	6.64 (0.38) 4.68 - 9.01	4.32	0.32 (0.24)	0.203
Total fat	2.82 (0.12) 2.17 - 3.63	2.69 (0.12) 1.56 - 3.53	1.97	0.12 (0.15)	0.473
Carbohydrates by calculation	86.19 (0.58) 83.16 - 88.79	86.98 (0.58) 82.69 - 89.53	6.84	-0.79 (0.41)	0.064
ADF	22.04 (0.67) 18.18 - 28.00	21.26 (0.67) 16.83 - 25.04	8.22	0.78 (0.70)	0.282
NDF	34.07 (0.81) 27.29 - 43.83	33.99 (0.81) 27.87 - 40.38	12.51	0.080 (1.08)	0.941
Ash	4.02 (0.26) 2.71 - 6.27	3.72 (0.26) 2.46 - 4.87	2.41	0.29 (0.20)	0.155

[1]dw = dry weight
[2]Mean (S.E.) = least-square mean (standard error) obtained from the linear mixed model analysis
[3]Maximum value minus minimum value for the control maize hybrid

Table VI-5. Literature and ILSI Database Ranges for Components in Maize Grain and Forage

Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Grain Nutrients		
Proximates		
protein (% dw)	8.27-13.33[a]; 9.17-12.19[b]	5.72-17.26
total fat (% dw)	2.95-4.40[a]; 3.18-4.23[b]	1.363-7.830
ash (% dw)	1.17-2.01[a]; 1.27-1.63[b]	0.616-6.282
Amino Acids		
alanine (% dw)	0.60-1.04[a]; 0.68-0.96[b]	0.40-1.48
arginine (% dw)	0.34-0.52[a]; 0.34-0.50[b]	0.12-0.71
aspartic acid (% dw)	0.52-0.78[a]; 0.59-0.76[b]	0.30-1.21
cystine (% dw)	0.19-0.26[a]; 0.20-0.26[b]	0.12-0.51
glutamic acid (% dw)	1.54-2.67[a]; 1.71-2.44[b]	0.83-3.54
glycine (% dw)	0.33-0.43[a]; 0.33-0.42[b]	0.184-0.685
histidine (% dw)	0.25-0.37[a]; 0.27-0.34[b]	0.14-0.46
isoleucine (% dw)	0.30-0.48[a]; 0.32-0.44[b]	0.18-0.69
leucine (% dw)	1.02-1.87[a]; 1.13-1.65[b]	0.60-2.49
lysine (% dw)	0.26-0.33[a]; 0.28-0.31[b]	0.129-0.668
methionine (% dw)	0.17-0.26[a]; 0.16-0.30[b]	0.11-0.47
phenylalanine (% dw)	0.43-0.72[a]; 0.45-0.63[b]	0.24-0.93
proline (% dw)	0.74-1.21[a]; 0.78-1.11[b]	0.46-1.75
serine (% dw)	0.39-0.67[a]; 0.43-0.6[b]	0.15-0.77
threonine (% dw)	0.29-0.45[a]; 0.31-0.39[b]	0.17-0.67
tryptophan (% dw)	0.047-0.085[a]; 0.042-0.07[b]	0.027-0.215
tyrosine (% dw)	0.13-0.43[a]; 0.12-0.41[b]	0.10-0.73
valine (% dw)	0.42-0.62[a]; 0.45-0.58[b]	0.27-0.86
Fatty Acids		
palmitic acid (% Total FA)	8.80-13.33[a]; 9.84-12.33[b]	6.81-26.55
palmitoleic acid (% Total FA)	0.059-0.23[a]	0.067-0.453
stearic acid (% Total FA)	1.36-2.14[a]; 1.3-2.1[b]	1.02-3.83
oleic acid (% Total FA)	19.50-33.71[a]; 19.59-29.13[b]	16.38-42.81
linoleic acid (% Total FA)	49.31-64.70[a]; 56.51-65.65[b]	34.27-67.68
linolenic acid (% Total FA)	0.89-1.56[a]; 1.03-1.38[b]	0.55-2.33
arachidic acid (% Total FA)	0.30-0.49[a]; 0.30-0.41[b]	0.267-0.993
eicosenoic acid (% Total FA)	0.17-0.29[a]; 0.17-0.27[b]	0.098-1.952
behenic acid (% Total FA)	0.069-0.28[a]; 0.059-0.18[b]	0.093-0.417
Carbohydrates By Calculation		
carbohydrates by calculation (% dw)	81.31-87.06[a]; 82.10-85.98[b]	77.4-89.7
Fiber		
ADF (% dw)	1.82-4.48[a]; 1.83-3.39[b]	1.41-11.34
NDF (% dw)	6.51-12.28[a]; 6.08-10.36[b]	4.28-24.30
TDF (% dw)	10.65-16.26[a]; 10.57-14.56[b]	5.78-35.31
Minerals		
calcium (% dw)	0.0036-0.0068[a]; 0.0035-0.007[b]	0.001-0.101

Table VI-5. Literature and ILSI Database Ranges for Components in Maize Grain and Forage (continued)

Tissue Components[1]	Literature Range[2]	ILSI Range[3]
copper (mg/kg dw)	0.85-3.54[c]	0.55-21.20
iron (mg/kg dw)	14.17-23.40[a]; 15.90-24.66[b]	9.51-191.00
magnesium (% dw)	0.091-0.14[a]; 0.1-0.14[b]	0.06-0.19
manganese (mg/kg dw)	4.83-8.34[a]; 4.78-9.35[b]	1.69-14.30
phosphorus (% dw)	0.24-0.37[a]; 0.27-0.38[b]	0.13-0.55
potassium (% dw)	0.29-0.39[a]; 0.36-0.43[b]	0.18-0.60
zinc (mg/kg dw)	16.78-28.17[a]; 18.25-30.44[b]	6.5-42.6
Vitamins		
vitamin A (mg/kg dw)	0.14-11.27[d]	0.19-80.20
vitamin B_1 (mg/kg dw)	2.33-4.17[a]; 2.71-4.33[b]	1.26-40.00
vitamin B_2 (mg/kg dw)	0.94-2.42[a]; 1.64-2.81[b]	0.50-7.35
vitamin B_3 (mg/kg dw)	15.07-32.38[a]; 13.64-42.06[b]	7.42-46.94
vitamin B_6 (mg/kg dw)	4.93-7.53[a]; 4.97-8.27[b]	1.18-12.14
vitamin B_9 (mg/kg dw)	0.19-0.35[a]; 0.23-0.42[b]	0.09-3.50
vitamin E (mg/kg dw)	5.96-18.44[a]; 2.84-15.53[b]	0.84-68.67
Grain Other		
Anti-Nutrients		
phytic acid (% dw)	0.69-1.09[a]; 0.60-0.94[b]	0.111-1.940
raffinose (% dw)	0.079-0.22[a]; 0.061-0.15[b]	0.020-0.466
Secondary Metabolites		
ferulic acid (µg/g dw)	1205.75-2873.05[a]; 1011.40-2539.86[b]	291.93-4397.30
p-coumaric acid (µg/g dw)	94.77-327.39[a]; 66.48-259.68[b]	53.4-820.0
Forage Nutrients		
Proximates		
protein (% dw)	5.80-10.24[a]; 5.56-9.14[b]	2.37-16.32
total fat (% dw)	1.28-3.62[a]; 0.20-1.76[b]	0.296-6.755
ash (% dw)	2.67-8.01[a]; 4.59-6.9[b]	0.66-13.20
Carbohydrates By Calculation		
carbohydrates by calculation (% dw)	81.88-89.26[a]; 84.11-87.54[b]	73.3-92.9
Fiber		
ADF (% dw)	19.11-30.49[a]; 20.73-33.39[b]	5.13-47.39
NDF (% dw)	27.73-49.62[a]; 31.81-50.61[b]	18.30-67.80
Minerals		
calcium (% dw)	0.12-0.33[a]; 0.21-0.41[b]	0.04-0.58
phosphorus (% dw)	0.090-0.26[a]; 0.13-0.21[b]	0.07-0.44

[1]dw = dry weight; FA = Fatty Acid; mg/kg/ dw

[2]Literature range references: [a](Harrigan et al., 2009) (see U.S. Field data);[b](Harrigan et al., 2009) (see Chile field data);[c](Ridley et al., 2011);[d](Egesel et al., 2003)

[3]ILSI range is from ILSI Crop Composition Database, version 7, 2019 (Accessed January 25th, 2019).

VII. ASSESSMENT OF POTENTIAL IMPACTS ON NON-TARGET ORGANISMS BENEFICIAL TO AGRICULTURE AND THREATENED AND ENDANGERED SPECIES

Evaluation of the potential risks to NTOs is an important component of the risk assessment of a biotechnology-derived crop. Since risk is a function of hazard and exposure, it is critical to determine the potential hazard as well as routes and levels of exposure. MON 95379 maize will not be commercialized in the United States. Under the proposed terms of an EPA seed increase registration application, MON 95379 maize will be cultivated for small-scale breeding, testing, and seed increase-related activities in three U.S. states including Nebraska, Hawaii, and Iowa with a maximum combined total acreage of 100 acres per growing season and small-scale confined field trials (potentially in other locations) to collect needed regulatory data to support global regulatory submissions of future products stacked with MON 95379. Environmental exposure to Cry1B.868 and Cry1Da_7 is expected to be extremely limited based on the combined total acreage and therefore, the cultivation of MON 95379 maize is unlikely to result in unacceptable risk to populations of NTOs beneficial to agriculture.

The results of Tier I toxicity testing with NTOs representing key ecological functions is summarized within this section. The risk assessment plan was informed by the mode of action (MOA) of the proteins, the spectrum of insecticidal activity for the Cry1B.868 and Cry1Da_7 proteins, the expression profile of these proteins in relevant tissues, the potential routes of exposure to NTOs, and the biology and familiarity with maize. A conceptual model was developed and identified species representing key ecological functions with relevant direct or indirect dietary exposure to Cry1B.868 and Cry1Da_7 proteins expressed in MON 95379 maize. An exposure assessment was conducted to calculate conservatively estimated environmental concentrations (EECs) relevant to NTOs representing key ecological functions identified in the conceptual model. The calculated EEC values were compared to endpoints from the laboratory NTO testing with Cry1B.868 and Cry1Da_7 proteins to calculate margins of exposure (MOE) and characterize risk. Finally, the potential risk of limited cultivation of MON 95379 maize to threatened and endangered species was evaluated.

VII.A. Mode of Action and Spectrum of Insecticidal Activity

Cry1B.868 and Cry1Da_7 both belong to the Cry1 family of insecticidal proteins, which have a long history of safe use (Wang et al., 2019). The mode of action of these proteins is similar to previously commercialized Cry proteins and involves the same general steps of ingestion, solubilization, oligomerization and formation of selective ion channels (commonly referred to as pore-formation), which leads to cell lysis in the midgut and ultimately mortality (Pigott and Ellar, 2007; Wang et al., 2019). All the steps mentioned above are required for biological activity to occur, hence the specific nature of the biological activity of Cry proteins. Cry1 family proteins have been expressed in crops for specific management of lepidopteran pests (Koch et al., 2015).

Cry1B.868 and Cry1Da_7 proteins were individually tested in initial laboratory screening assays against a range of agronomically and ecologically relevant taxa to characterize their spectrum of insecticidal activity (Appendix L). Diet incorporation bioassays for test organisms were conducted and these bioassays were designed to: (1) provide continuous exposure of each protein to the test

species and (2) provide a sufficient duration of exposure to evaluate the potential effects of each protein on survival. Proteins were provided in diet at or above expected concentrations in the highest expressing tissue in MON 95379 plants (i.e., early-stage leaf). Details regarding general methodologies for activity spectrum assays have been published previously (Bachman et al., 2013). While Monarch butterfly (*Danaus plexippus*) was not tested previously, the Monarch butterfly activity spectrum tests conducted with Cry1B.868 and Cry1Da_7 were based on the methodologies of lepidopteran tests described in the Bachman publication.

The results from bioassays with three lepidopteran insect pests, fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*), and European corn borer (*Ostrinia nubilalis*) indicate that Cry1B.868 and Cry1Da_7 proteins are active against these lepidopteran pest species (Table VII-1 and Table VII-2). Biological activity was also detected in the other lepidopteran species evaluated, Monarch butterfly. No toxic effects were observed at the highest dose tested in the activity spectrum evaluations in species outside of Lepidoptera, including coleopteran species western corn rootworm (*Diabrotica virgifera virgifera*), southern corn rootworm (*Diabrotica undecimpunctata howardi*), Colorado potato beetle (*Leptinotarsa decimlineata*), or Mexican bean beetle (*Epilachna varivestis*), or hemipteran species of western tarnished plant bug (*Lygus hesperus*) and neotropical brown stink bug (*Euschistus heros*). The results of these initial screening studies indicated that the spectrum of activity of Cry1B.868 and Cry1Da_7 was limited to Lepidoptera.

Table VII-1. Activity Spectrum Results from Feeding Assays with Cry1B.868 Protein in Invertebrates

Order	Family	Genus Species	Representative Function	EC_{50} or LC_{50} (µg/mL diet)	Activity
Lepidoptera	Noctuidae	*Spodoptera frugiperda*	Herbivore	0.15	Yes[b]
Lepidoptera	Noctuidae	*Helicoverpa zea*	Herbivore	120	Yes[b]
Lepidoptera	Crambidae	*Ostrinia nubilalis*	Herbivore	9.8	Yes[b]
Lepidoptera	Nymphalidae	*Danaus plexippus*	Herbivore	0.077	Yes[c]
Coleoptera	Chrysomelidae	*Diabrotica virgifera virgifera*	Herbivore	>301[a]	No[d]
Coleoptera	Chrysomelidae	*Diabrotica undecimpunctata howardi*	Herbivore	>837[a]	No[d]
Coleoptera	Chrysomelidae	*Leptinotarsa decemlineata*	Herbivore	>1708[a]	No[d]
Coleoptera	Coccinellidae	*Epilachna varivestis*	Herbivore	>837[a]	No[d]
Hemiptera	Miridae	*Lygus hesperus*	Herbivore	>700[a]	No[e]
Hemiptera	Pentatomidae	*Euschistus heros*	Herbivore	>700[a]	No[f]

[a] The most conservative (i.e. lowest) concentration observed at the highest treatment level is reported based on diet stability analysis conducted at multiple times during exposure. The measured concentrations were above the 95th percentile values of Cry1B.868 protein expression (µg/g fw) in MON 95379 leaf tissue (Table VII-3).
[b] EC_{50} value was estimated in a 7-day exposure to Cry1B.868 protein treated diets for ≥5 concentrations.
[c] LC_{50} value was estimated in a 7-day exposure to Cry1B.868 protein treated diets for 7 concentrations.
[d] Activity was measured for survival in a 7-day feeding exposure to Cry1B.868 protein.
[e] Activity was measured for survival in a 6-day feeding exposure to Cry1B.868 protein.
[f] Activity was measured for survival in a 5-day feeding exposure to Cry1B.868 protein.

Table VII-2. Activity Spectrum Results from Feeding Assays with Cry1Da_7 Protein in Invertebrates.

Order	Family	Genus Species	Representative Function	EC_{50} or LC_{50} (µg/mL diet)	Activity
Lepidoptera	Noctuidae	*Spodoptera frugiperda*	Herbivore	0.096	Yes[b]
Lepidoptera	Noctuidae	*Helicoverpa zea*	Herbivore	0.042	Yes[b]
Lepidoptera	Crambidae	*Ostrinia nubilalis*	Herbivore	11	Yes[b]
Lepidoptera	Nymphalidae	*Danaus plexippus*	Herbivore	0.016	Yes[c]
Coleoptera	Chrysomelidae	*Diabrotica virgifera virgifera*	Herbivore	>106[a]	No[d]
Coleoptera	Chrysomelidae	*Diabrotica undecimpunctata howardi*	Herbivore	>65[a]	No[d]
Coleoptera	Chrysomelidae	*Leptinotarsa decemlineata*	Herbivore	>58[a]	No[d]
Coleoptera	Coccinellidae	*Epilachna varivestis*	Herbivore	>65[a]	No[d]
Hemiptera	Miridae	*Lygus hesperus*	Herbivore	>50[a]	No[e]
Hemiptera	Pentatomidae	*Euschistus heros*	Herbivore	>50[a]	No[f]

[a] The most conservative (i.e. lowest) concentration observed at the highest treatment level is reported based on diet stability analysis conducted at multiple times during exposure. The measured concentrations were above the 95th percentile values of Cry1Da_7 protein expression (µg/g fw) in MON 95379 leaf tissue (Table VII-3).
[b] EC_{50} value was estimated in a 7-day exposure to Cry1Da_7 protein treated diets for ≥5 concentrations.
[c] LC_{50} value was estimated in a 7-day exposure to Cry1Da_7 protein treated diets for 7 concentrations.
[d] Activity was measured for survival in a 7-day feeding exposure to Cry1Da_7 protein.
[e] Activity was measured for survival in a 6-day feeding exposure to Cry1Da_7 protein.
[f] Activity was measured for survival in a 5-day feeding exposure to Cry1Da_7 protein.

VII.B. Exposure Assessment

MON 95379 maize will not be commercialized in the United States. Under the proposed terms of an EPA seed increase registration application, cultivation of MON 95379 maize will be limited to three U.S. states, including Nebraska, Hawaii, and Iowa, with a maximum combined acreage of 100 acres per growing season and small-scale, confined field trials in additional locations to collect needed regulatory data to support global regulatory submissions of future products stacked with MON 95379. Therefore, environmental exposure to Cry1B.868 and Cry1Da_7 is expected to be extremely limited and the cultivation of MON 95379 maize is unlikely to result in adverse effects to NTO populations.

An exposure assessment was conducted, and illustrated in Figure VII-1, to characterize potential routes of exposure of Cry1B.868 and Cry1Da_7 proteins expressed in MON 95379 maize to NTOs. Direct exposure to NTOs is expected to be through consumption of maize tissues (e.g. leaf, pollen, grain) expressing the Cry1B.868 and Cry1Da_7 proteins (Figure VII-1). The conceptual model identified pollinators, facultative predators and parasitoids, wild and managed vertebrates and organisms involved in decomposition and soil nutrient cycling as species with relevant direct or indirect dietary exposure to Cry1B.868 and Cry1Da_7 proteins expressed in MON 95379 maize. Only non-pest species were considered relevant for an assessment of invertebrate herbivores (e.g. Monarch butterfly). Cry proteins are not known to possess contact toxicity in sensitive or non-target species and this route of exposure was not considered further.

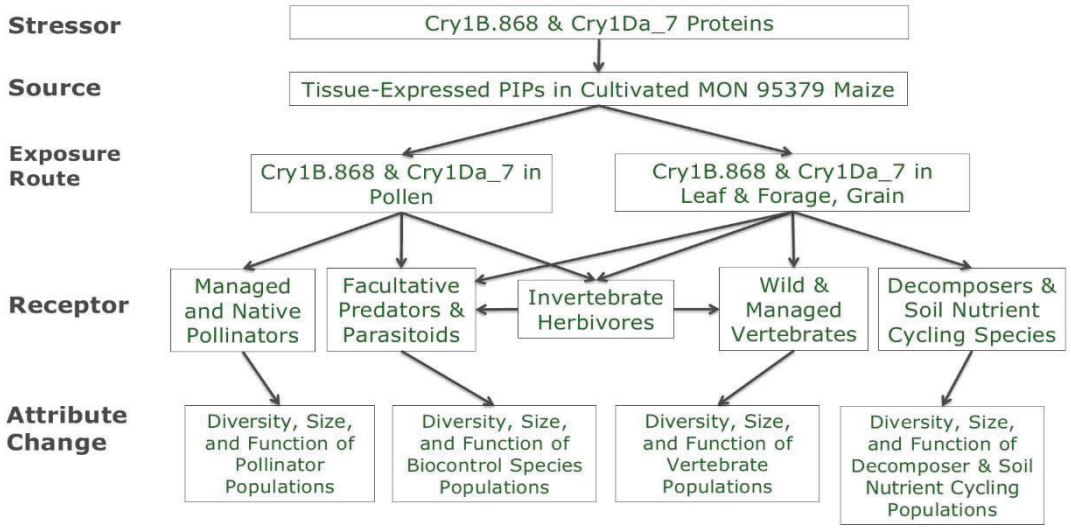

Figure VII-1. Relevant Routes of Exposure to Non-Target Organisms from Cultivation of MON 95379 Maize

The above conceptual model depicts relevant routes of exposure to non-target organisms from cultivation of MON 95379 maize. Relevant tissues expressing Cry1B.868 and Cry1Da_7 proteins were identified as pollen, leaf, forage, and grain. Key non-target organism groups with expected direct or indirect exposure to Cry1B.868 and Cry1Da_7 include pollinators, facultative predators and parasitoids, wild and managed vertebrate species and organisms involved in decomposition

and soil nutrient cycling. Only non-pest species were consider relevant for an assessment of invertebrate herbivores (e.g., monarch butterfly).

For the exposure assessment described below, conservatively estimated environmental concentration (EEC) values were calculated to characterize exposure to NTOs and were based on expression of Cry1B.868 and Cry1Da_7 evaluated in multiple tissues and growth stages. For tissue types where multiple growth stages were analyzed, the growth stage with highest mean expression was identified and the upper 95th percentile fresh weight expression values were then calculated. Maximum predicted environmental concentrations of Cry1B.868 and Cry1Da_7 proteins in soil were calculated based on a soil incorporation model and soil dissipation results. Finally, Food Intake Rate (FIR) and the concetrations of the proteins in diet were used to estimate dietary exposure to Cry1B.868 and Cry1Da_7 to terrestrial vertebrates such as birds and small mammals.

VII.B.1. Expression of Cry1B.868 and Cry1Da_7 in MON 95379 Tissues

The expression of Cry1B.868 and Cry1Da_7 in MON 95379 maize was measured with lyophilized tissue and reported on a dry weight basis (Section V.F). Since fresh tissue is most relevant for exposure to NTOs, a conversion factor was used to calculate the amount of Cry1B.868 and Cry1Da_7 in fresh weight tissues and the 95th percentiles of the mean fresh weight expression values were used to provide conservative EEC values for ecological exposure. These values served as the basis for high-end estimates of exposure levels of Cry1B.868 and Cry1Da_7 to target pests and non-target organisms. Given that the highest expression levels for both Cry1B.868 and Cry1Da_7 were measured in V2-V4 stage leaves (OSL1) of MON 95379 maize, all concentrations tested in the activity spectrum assessment (Table VII-1 and Table VII-2) were based upon 20.9 µg Cry1Da_7/g fresh weight and 111.4 µg Cry1B.868/g fresh weight leaf tissue (Table VII-3). In addition to leaf tissue, the 95th percentile of fresh weight expression levels of Cry1B.868 and Cry1Da_7 in tissues deemed most relevant to NTO exposure (e.g. pollen, forage, and grain) were also determined (Table VII-3). Levels of Cry1Da_7 could not be quantified in pollen and therefore the assay LOQ (0.125 µg Cry1Da_7/g dw pollen) was used as worst-case estimate of exposure to Cry1Da_7 via pollen.

Table VII-3. Fresh Weight 95th Percentile Expression of Cry1Da_7 and Cry1B.868 Proteins in Maize Tissues Collected from MON 95379 Produced in United States Field Trials in 2018

Tissue Type[a]	Development Stage[b]	Cry1Da_7 (µg/g fw)[c]	Cry1B.868 (µg/g fw)[c]
OSL1	V2-V4	20.9	111.4
pollen	R1	<LOQ[d]	61.9
forage	R5	11.1	48.8
grain	R6	0.5	41.2

[a]OSL = over season leaf
[b]The crop development stage at which each tissue was collected. In cases where multiple development stages were analyzed, the growth stage with the maximum observed value was used for calculating EECs.
[c]Protein levels are expressed as the 95th percentile of the mean expression expressed in microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw).
[d]LOQ = limit of quantitation. The LOQ for Cry1Da_7 in pollen was 0.125 µg/g dry weight.

VII.B.2. Estimated Environmental Concentrations of Cry1B.868 and Cry1Da_7

Predators and Parasitoids

The concentrations of Cry1B.868 and Cry1Da_7 in leaves taken from plants in the V2-V4 growth stage were used to determine potential exposure of predator and parasitoids to Cry1B.868 and Cry1Da_7. Growth stage V2-V4 leaf was the stage and tissue-type with the highest observed expression for both proteins and serves as a worst-case exposure level. The expression values were 20.9 µg Cry1Da_7 and 111.4 µg Cry1B.868 per gram of fresh weight leaf tissue (Table VII-3). For predators and parasitoids, the most relevant exposure scenario is predation of herbivore prey exposed to MON 95379 tissues. The degree of such exposure would be predicated on the concentrations of Cry1B.868 and Cry1Da_7 acquired in prey species. Herbivores generally contain lower levels of Cry proteins than what is expressed in the tissues being consumed. Minimal concentrations of Cry proteins have been found in aphids and other phloem-feeding insects (Obrist et al., 2006); reviewed in Romeis et al. (2014). Lepidopteran pest larvae and thrips have been shown to contain between 0.1 and 0.35 times the amount of Cry1Ab protein expressed in maize tissue (reviewed in (Raybould et al., 2007)). The highest concentrations of Cry protein found in herbivores have been observed in spider mites where observed levels vary, but may be similar to those expressed in maize leaf tissues (Obrist et al., 2006).

Given the broad diversity of prey taxa likely to be consumed by beneficial predators in the agro-ecosystem, leaf expression levels for Cry1B.868 and Cry1Da_7 are sufficiently conservative to

account for indirect exposure to generalist predators feeding on herbivore prey (Table VII-4; (U.S. EPA, 2016; Appendix L). Leaf expression is likely a very conservative estimate of indirect exposure through prey and previously published risk assessments have proposed a value of 0.2-times leaf expression as a more realistic estimate of prey exposure to predators and parasitoids (Raybould et al., 2007). Some predators and parasitoids will also consume pollen and nectar to supplement nutrition during specific stages in development or when preferred prey is scarce (Li et al., 2008; Li et al., 2010). As maize does not produce nectar, 95th percentile expression values for Cry1B.868 and Cry1Da_7 in pollen were used to estimate this route of exposure (Table VII-4).

Table VII-4. Summary of Estimated Environmental Concentrations (EECs) for Exposure of Invertebrate NTOs to Cry1B.868 and Cry1Da_7 in MON 95379 Maize

NTO Group	Test Species	Exposure route used to derive EEC value	Cry1B.868 EEC (µg/g[d])	Cry1Da_7 EEC (µg/g[d])
Predators & Parasitoids	*Coleomegilla maculata*	Diet 100% MON 95379 leaves[a]	111.4	20.9
	Poecilus cupreus	Diet 100% MON 95379 leaves	111.4	20.9
	Geocoris punctipes	Diet 100% MON 95379 leaves	111.4	20.9
	Pediobius foveolatus	Diet 100% MON 95379 leaves	111.4	20.9
	Chrysoperla sp. (larva)	Diet 100% MON 95379 leaves	111.4	20.9
	Chrysoperla sp. (adult)	Diet 100% MON 95379 pollen	61.9	0.1[b]
Soil organisms	*Folsomia candida*	Soil incorporation of MON 95379 forage	0.4	0.2[c]
	Eisenia andrei	Soil incorporation of MON 95379 forage	0.4	0.2[c]
Pollinators	*Apis mellifera* (adult)	Diet 100% MON 95379 pollen	61.9	0.1[b]
	Apis mellifera (larva)	Diet 100% MON 95379 pollen	61.9	0.1[b]

[a]For beneficial predator and parasitoids where herbivore prey is the most relevant route of exposure, 95th percentile leaf expression was used as a worst-case exposure level.
[b]Cry1Da_7 could note be quantified in pollen so the LOQ (0.125 µg/g) was used to derive the EEC.
[c] Calculation of EEC values for Cry1B.868 and Cry1Da_7 are described in the section on soil invertebrates and are derived from PEC values (Table VII-5). The EEC for Cry1Da_7 was derived from the maximum predicted concentration assuming a DT_{50} of 1 year in sandy loam soil (Table VII-6).
[d]Values expressed as µg protein per g of tissue or soil.

Non-target Lepidoptera

The spectrum of activity for Cry1B.868 and Cry1Da_7 was limited to Lepidoptera and therefore consideration was given to potential exposure to non-target lepidopteran species. Non-target lepidopteran (i.e., non-pest) species are generally not known to feed on maize tissue as their feeding and reproductive ecology is typically tightly associated with a preferred host plant (U.S. EPA, 2001a). Standard agricultural practices for weed management would typically minimize the growth of such host plants within the boundaries of the maize field. Therefore, exposure to non-target lepidopteran species would most likely be through the off-crop movement and deposition of pollen on preferred host plants adjacent to the field. Previous work with maize pollen and deposition on milkweed, a host plant for monarch butterfly larvae, has demonstrated that density of pollen deposition decreases exponentially with increasing distance from the field edge. Pleasants et al. (2001) reported that at 4 to 5 meters from the edge of a maize field, the maize pollen density on milkweed leaves was expected to be less than 25 grains per cm^2 95% of the time; a density less than 10% of the 95^{th} percentile measured at the field edge. Based on these results, at 10 meters from the field edge, expected density of deposition would be less than 2 grains per cm^2 indicating there is essentially no exposure to pollen beyond 10m. Standard agronomic management practices commonlyutilized for the small-scale field plantings of MON 95379 will result in negligible pollen exposure to non-target Lepidoptera (see Appendix L.21).

Under the proposed terms of an EPA seed increase registration application, MON 95379 maize will be cultivated for small-scale breeding, testing, and seed increase-related activities in three U.S. states including Nebraska, Hawaii, and Iowa with a maximum combined total acreage of 100 acres per growing season (as well as potential small-scale confined field trials for stack products in other locations, as described earlier). Cultivation of MON 95379 under the terms of the proposed EPA registration minimizes the potential exposure of MON 95379 pollen to non-target Lepidoptera populations. Furthermore, the extent of off-crop movement of maize pollen is limited temporally as peak production of pollen from a maize field typically occurs within a 4-day interval (Abendroth et al., 2011). In addition, wind and rain events during or following pollen shed can significantly reduce pollen densities on non-target lepidopteran host plants near the field. In a combined analysis of six pollen deposition trials across four regions where maize is cultivated, at least one rainfall event occurred during anthesis in all but one trial and these rain events resulted in 54-86% reduction in densities of pollen deposition (Pleasants et al., 2001). Analysis of prevailing wind patterns also indicated the potential exposure from off-crop pollen deposition was limited to the downwind side of the field. Standard agronomic practices used to produce high quality seed/data in breeding and seed increase nurseries (e.g., weed control and buffer rows) further limit movement of MON 95379 pollen off-crop and minimize exposure to non-target Lepidoptera. Based on these reasons, minimal off-crop exposure of non-target Lepidoptera to Cry1B.868 and Cry1Da_7 in MON 95379 pollen is expected and this exposure is further minimized by the limited acreage for intended cultivation. Therefore, the expected absence of significant exposure of MON 95379 pollen supports the conclusion of minimal risk to non-target Lepidoptera.

Pollinators

Exposure of species responsible for pollination services to Cry proteins is typically the result of pollinators foraging for pollen and nectar in crops expressing the Cry protein of interest. The honey bee (*Apis mellifera*) serves as a representative species for protecting bee diversity and pollination services as honey bees will forage nectar and pollen for nutrition and support of the hive and developing larva. Since maize does not produce nectar, exposure to honey bees is limited to the presence of Cry1B.868 and Cry1Da_7 in pollen. Analysis of expression data indicated that the 95th percentile concentration of Cry1B.868 in pollen is 61.9 µg/g pollen. Cry1Da_7 was not quantifiable by ELISA in pollen and therefore the assay LOQ value of 0.125 µg/g pollen was used as a worst-case estimate (Table VII-4).

Soil Invertebrates

The most relevant route of exposure for MON 95379 to soil invertebrates is via tillage of late season maize tissue into the top 6 inches of soil (Appendix L). To calculate predicted environmental concentrations (PECs) of Cry1B.868 and Cry1Da_7 in soil, the amount of biomass (stover) expected to remain in the field following cultivation and harvest of maize was determined. The mass of maize stover biomass is related to yield by harvest index and the relationship is defined as:

$$Harvest\ Index = \frac{Grain_{lbs}}{(Grain_{lbs} + Stover_{lbs})}$$

Using the 5-year national average for grain yield in the United States from 2014-2018 (173.4 Bu/A) and a typical harvest index value of 0.5 for maize results in a value of 4408.5 kg stover biomass/acre (Chakwizira et al., 2016; Ertl, 2013; Fischer et al., 2020; Ion et al., 2015; Li et al., 2015; Zwahlen et al., 2003; Zwahlen et al., 2007). For late season tissue, 95th percentile expression in forage was used to calculate the PEC values for Cry1B.868 and Cry1Da_7 in soil (Table VII-3) as expression of these proteins in forage tissue was about 5-fold higher than that found in forage root. Assuming incorporation of the entire calculated post-harvest biomass into the top 6 inches of soil with a bulk density of 1 g/cm^3, the 1× field load was calculated to be 7.3 g fw tissue/kg soil, and based on expression levels in forage tissue, soil concentrations for Cry1B.868 and Cry1Da_7 were calculated to be 0.35 µg/g soil and 0.08 µg/g soil respectively (Table VII-5).

Table VII-5. Predicted Environmental Concentrations (PECs) and Dissipation Kinetics of Cry1B.868 and Cry1Da_7 in Soil

Parameter	Soil Type	Cry1B.868	Cry1Da_7
PEC (µg protein/g soil)	n.a.[a]	0.35	0.08
DT_{50} (d)[a]	Sandy Loam	19	>213[b]
DT_{90} (d)	Sandy Loam	49	>213[b]
DT_{50} (d)	Silt Loam	9	40
DT_{90} (d)	Silt Loam	24	>213[b]
DT_{50} (d)	Clay Loam	8	36
DT_{90} (d)	Clay Loam	28	>213[b]

[a]n.a. = not applicable; DT_x = X% dissipation time
[b]The dissipation parameter could not be determined as it fell outside of the exposure period of the study. The Cry1Da_7 protein concentrations remaining at the end of the study expressed as percentage of maximum protein concentrations detected were 66%, 25%, and 22% in Sandy Loam, Silt Loam, and Clay Loam soils respectively.

Soil organisms may be exposed to the Cry1B.868 and Cry1Da_7 proteins by incorporation of above ground plant tissues (stover) into soil after harvest, and to a much lesser extent, by pollen deposited on the soil, or by root exudation. Exposure may occur by feeding on incorporated maize biomass or by ingestion or absorption of the Cry proteins to soil particles after their release from maize tissues into the soil. Clay soils, often associated with higher cation-exchange capacities, can generally bind macromolecules such as Cry proteins and nucleic acids more tightly than other soil types like sand and silt (Gruber et al., 2012; Icoz and Stotzky, 2008). Binding to clay particles can render proteins less available to enzymatic degradation and result in prolonged dissipation. However, other factors can also influence dissipation rates including protein characteristics, organic matter content, robustness of the microbial communities, soil moisture, and pH. Given the variety of environmental factors that influence degradation, it is not unexpected that relative degradation rates across soil types would vary across studies and proteins. Generally, microbial activity is thought to be a principle factor in degradation rates of free protein (Icoz and Stotzky, 2008). Radiolabeled ^{14}C-Cry1Ab applied to soils resulted in rapid CO_2 evolution and the ^{14}C-label was incorporated into the microbial biomass indicating that the applied Cry1Ab protein was being utilized as a microbial nutrition source (Valldor et al., 2015). This result is consistent with the notion that proteins are typically amendable to biodegradation in the environment. Guidance from EPA test guideline OPPTS 835.4100 recommends that soil dissipation tests generally not be conducted beyond 120 days due to the potential decline of microbial communities in test systems that are isolated from the environment and natural replenishment. For this reason, laboratory studies represent a more conservative approach to assess the potential for persistence of Cry

proteins in soils. Indeed, published field monitoring studies with other *Bt* crops have shown no persistence or accumulation of Cry proteins in fields where corn (Cry1Ab, Cry1F, Cry3Bb1) or cotton (Cry1Ac, Cry2Ab2) were grown for one or more years (Ahmad et al., 2005; Dubelman et al., 2005; Gruber et al., 2012; Head et al., 2002; Shan et al., 2008). In a comprehensive multi-year field survey, Gruber and colleagues evaluated soils for the presence of Cry1Ab in fields that were planted with MON 810 maize for nine consecutive years (Gruber et al., 2012). Over the course of the 9-year trial, Cry1Ab was detected in soil above detectable levels at a single site 6 weeks after the 8th consecutive growing season. Notably, no Cry1Ab protein was detected at any site in any of the spring samplings prior to planting indicating that seasonal decreases in soil temperature did not result in persistence or accumulation of Cry1Ab in soil. The predicted environmental concentrations of Cry1B.868 and Cry1Da_7 in soil are 0.35 and 0.08 µg/g soil, respectively (Table VII-5). With cultivation of MON 95379 maize in the U.S. limited to a maximum of 100 acres per growing season, this further supports a conclusion of minimal exposure of Cry1B.868 and Cry1Da_7 to soil organism populations. Further discussion of the fate of Cry proteins in soil can be found in Appendix L.

Although exposure is limited by the small area of intended planting, a study was conducted to estimate the dissipation rate of Cry1B.868 and Cry1Da_7 in agricultural soils (Appendix J). Lyophilized and ground MON 95379 leaf tissue, collected at the V2-V4 growth stage, was incorporated into agriculturally relevant soils at a rate of 75 mg/g soil (75 g/kg soil). This rate is approximately 10-fold higher than the 1× field load (7.3 g fw tissue/kg soil) calculated above. Soil microcosms were incubated in the dark at approximately 22 °C for 213 days and samples were collected at multiple sampling times during the duration of the study. The concentrations of Cry1B.868 and Cry1Da_7 proteins in collected soil samples were measured with specific ELISA assays. In a complimentary analysis, the temporal change in the biological activity of incorporated MON 95379 maize tissue in collected soil samples was evaluated using a sensitive bioassay with *Spodoptera frugiperda* (fall armyworm).

Results from the ELISA analysis indicated that Cry1B.868 dissipated rapidly in soils with DT_{50} values of 19, 9, and 8 days in sandy loam, silt loam, and clay loam soils respectively (Table VII-5). Calculated DT_{90} values for Cry1B.868 in soil were determined to be 49, 24, and 28 days in sandy loam, silt loam, and clay loam, respectively, indicating that Cry1B.868 is unlikely to persist or accumulate in agricultural soils. Therefore, the EEC for Cry1B.868 (Table VII-4) was the same as the PEC (Table VII-5) rounded to one significant digit.

Cry1Da_7 demonstrated a more prolonged dissipation profile compared to Cry1B.868. The DT_{50} values for Cry1Da_7 were determined to be 40 and 36 days in silt loam and clay loam soils, respectively. In sandy loam soil, the DT_{50} was not captured in the study (>213d) and the percent of maximum protein concentration remaining on day 213 was 66%, though it should be noted that the percent of maximum protein concentration remaining at the penultimate sampling time (121d) was 46% (Appendix L). Ninety percent dissipation of Cry1Da_7 was not reached in any soil type within the 213 day duration of the study. The relatively prolonged dissipation profile of Cry1Da_7 is supported by results with the insect bioassay that showed a gradual loss of biological activity over time.

Since a DT_{50} value could not be determined for Cry1Da_7 in sandy loam soil, a first-order soil accumulation model was used to estimate the effect of potential accumulation of Cry1Da_7 if

MON 95379 maize was grown once a year in the same location (FOCUS, 1997) without rotation. The model assumed a single planting per year, and a starting concentration of 0.08 µg Cry1Da_7/g soil. The model was then run under three different assumptions for the DT_{50} of Cry1Da_7 in sandy loam soil: 183d (1/2y), 274d (3/4y), and 365d (1y). An estimated DT_{50} of 365d in sandy loam soil is expected to be highly conservative as the observed DT_{50} values for Cry1Da_7 in silt loam and clay loam soils were 40 and 36 days respectively, greater than 50% dissipation was observed at day 121 in sandy loam soil, and no biological activity was detected in sandy loam soil samples containing MON 95379 maize tissue at the final sampling time of 213 days. The first order soil accumulation model is given by:

$$C(t) = PEC_0 + C(t-1)e^{(-\ln(2)/(\frac{DT50}{365})}$$

C(t) = amount of Cry1Da_7 protein remaining after soil incorporation in year *t*

PEC_0 = 0.08 µg Cry1Da_7/g soil

DT50 = DT_{50} in days

Under the highly conservative scenario assuming MON 95379 maize is planted in the same field without rotation for 25 consecutive years and using the most conservative estimated DT_{50} value, the soil accumulation model indicates that peak soil concentrations of Cry1Da_7 would plateau at about 2-fold above the initial PEC value for Cry1Da_7 in soil (Figure VII-2; Table VII-6). Therefore, the Cry1Da_7 EEC for soil organisms was conservatively calculated to be 0.16 µg/g soil; or 2× the initial PEC value for Cry1Da_7 in soil (Table VII-4).

Table VII-6. First-Order Accumulation Model to Predict Maximum Expected Concentration of Cry1Da_7 Protein in Sandy Loam Soil

	Cry1Da_7 (µg/g soil)				Cry1Da_7 (µg/g soil)		
Year	DT50 = 365d	DT50 = 274d	DT50 = 183d	Year	DT50 = 365d	DT50 = 274d	DT50 = 183d
1	0.08	0.08	0.08	14	0.16	0.13	0.11
2	0.12	0.11	0.10	15	0.16	0.13	0.11
3	0.14	0.12	0.11	16	0.16	0.13	0.11
4	0.15	0.13	0.11	17	0.16	0.13	0.11
5	0.16	0.13	0.11	18	0.16	0.13	0.11
6	0.16	0.13	0.11	19	0.16	0.13	0.11
7	0.16	0.13	0.11	20	0.16	0.13	0.11
8	0.16	0.13	0.11	21	0.16	0.13	0.11
9	0.16	0.13	0.11	22	0.16	0.13	0.11
10	0.16	0.13	0.11	23	0.16	0.13	0.11
11	0.16	0.13	0.11	24	0.16	0.13	0.11
12	0.16	0.13	0.11	25	0.16	0.13	0.11
13	0.16	0.13	0.11				

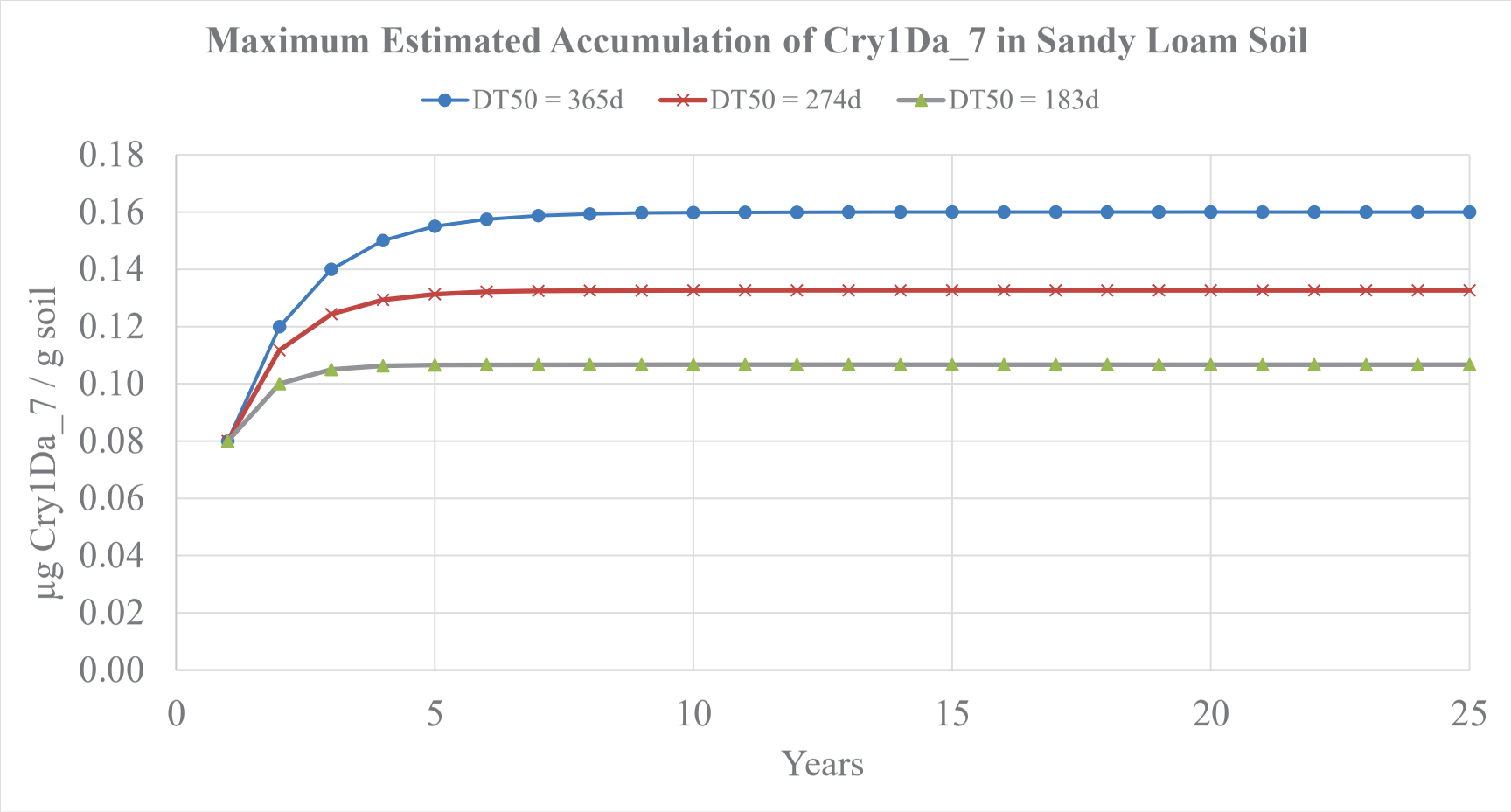

Figure VII-2. A First-Order Soil Accumulation Model Based on Highly Conservative Parameter Estimates Predicts Limited Accumulation of Cry1Da_7 in Sandy Loam Soil

Assuming a single yearly planting of MON 95379 and a conservative DT_{50} estimate of 1 year for Cry1Da_7 in sandy loam soil indicates that soil concentrations would peak at 2× the initial soil PEC value.

The observed dissipation kinetics for Cry1Da_7 are generally similar to those of Cry1Ac in MON 87701 soybean, which had DT_{90} values in silt and sandy soils that exceeded 181d. However, field monitoring surveys have demonstrated that Cry1 proteins, including Cry1Ac, do not persist or accumulate in the environment (Dubelman et al., 2005; Gruber et al., 2012; Head et al., 2002). This is likely due to additional factors and abiotic stressors present in the environment that include variations in temperature, rain events, exposure to sunlight as well as potentially higher numbers of microorganisms in field soil that could promote protein degradation. This is consistent with previous conclusions from EPA (U.S. EPA, 2001a):

> *"Field deposition of Cry protein would be associated with plant material (pollen or crop residue) or plant root exudates (e.g. carbohydrates and amino acids) which typically stimulate microbial activity and reproduction (Cheng and Coleman 1990, Griffiths et al. 1999, Jensen and Soerensen 1994, Meharg 1994). Many of the experiments examining persistence of Cry proteins reported in the published literature have apparently been conducted in bulk soils or soil components. Bulk soil generally does not support populations of microorganisms as high as in the rhizosphere or where plant residues are incorporated into the soil. Other work suggests typical ratios of 5-20 for rhizosphere to bulk soil microbes, with rhizosphere populations commonly 100 fold higher than in bulk soil (Atlas and Bartha 1993). Therefore, degradation rates under field conditions may be higher than those shown in bulk soil experiments."*

Even if there is some persistence of Cry1Da_7, ultimate soil concentrations are not expected to result in adverse effects to soil organisms and this conclusion is consistent with previous statements by EPA SAP (U.S. EPA, 2001c):

> *"Nevertheless, it must be emphasized that accumulation and even long-term persistence of Cry proteins in soil does not necessarily result in adverse environmental effects."*

Taken together, it is unlikely that Cry1B.868 and Cry1Da_7 will persist or accumulate in the environment and any potential accumulation of Cry1Da_7 would result in, at maximum, a 2-fold relative increase in soil concentrations. Despite the potential for limited accumulation of Cry1Da_7 in soil, cultivation of MON 95379 in the U.S. limited to the proposed maximum of 100 acres per growing season further supports a conclusion of minimal exposure of Cry1B.868 and Cry1Da_7 to soil organism populations.

Terrestrial Vertebrates

Wild birds are not known to appreciably feed on maize leaves and therefore would most likely be exposed to Cry1B.868 and Cry1Da_7 by consuming MON 95379 grain and recently germinated seedlings. Birds such as crows (*Corvus brachyrhynchos*), grackles (*Quiscalus quiscula*), and sandhill cranes (*Grus canadensis*) will uproot and feed on recently germinated maize seedlings (Blackwell et al., 2001; Raybould et al., 2007; Sterner et al., 2003). Blackbirds will feed on developing kernels as well as spilled kernels in maize fields (Linz et al., 2003; Raybould et al., 2007). Similarly, the primary route of exposure of Cry1B.868 and Cry1Da_7 to wild mammals is through consumption of MON 95379 maize kernels. Rodent species including ground squirrels

(*Spermophilus tridecemlineatus*), deer mice (*Peromyscus maniculatus*), house mice (*Mus musculus*), and voles (*Microtus* spp.) are known to feed on germinating maize seeds (Raybould et al., 2007; Sterner et al., 2003). Indirect exposure to wild birds and mammals could occur via predation of arthropod herbivores that have fed on MON 95379 maize tissues. However, the concentrations in herbivore prey are typically less than levels found in leaf tissue (discussed above in *Predators and Parasitoids*; (Raybould et al., 2007). Therefore, direct exposure to terrestrial herbivore vertebrates via consumption of leaf tissue is sufficiently conservative to account for indirect exposure from preying on herbivores that have consumed MON 95379 leaf tissue.

Since endpoints from Tier I toxicity tests in birds and mammals are normalized to body weight, a diet-based exposure normalized to body weight was calculated for birds and mammals. Consumption of MON 95379 germinating seedlings results in potential exposure to grain and vegetative tissues. Therefore, potential exposures of Cry1B.868 and Cry1Da_7 to wild birds and mammals representing herbivore and granivore groups were determined. Food Intake Rate (FIR) values for birds and mammals of different classes of body weights were calculated using the empirically derived equations (U.S. EPA, 1993):

Avian food consumption: $\mathrm{FIR} = \frac{(0.648 \times \mathrm{bw})^{0.651}}{(1-\mathrm{W})}$

and

Mammalian food consumption: $\mathrm{FIR} = \frac{(0.621 \times \mathrm{bw})^{0.564}}{(1-\mathrm{W})}$

where:

FIR = food intake rate in grams of fresh weight per day (g/day)

bw = body mass of animal (g)

W = mass fraction of water in food (U.S. EPA value = 0.8 for herbivores and 0.1 for granivores)

These calculations are the same that are used to estimate FIRs for avian and small mammal species in the current version of EPA's Terrestrial Residue EXposure model[12] (T-REX v1.5.2). The calculated FIR values were multiplied by the 95th percentile fresh weight expression values for Cry1B.868 and Cry1Da_7 in leaf and grain tissue (Table VII-3) to determine diet-based exposure levels to birds and small mammals (Table VII-7). These values are likely highly conservative as they assume 100% of an animal's diet consists of MON 95379 maize. Previous studies have indicated that maize kernels can comprise up to 50% of blackbird and grackle diets and 73% of wild rodent diets (Ellis et al., 1998; Homan et al., 1994; Houtcooper, 1978; McNichol et al., 1979; Raybould et al., 2007).

[12] https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/t-rex-version-15-users-guide-calculating-pesticide#Section3_1_1 (accessed June 29, 2020).

Table VII-7. Summary of Dose-Based Estimated Environmental Concentrations (EECs) for Exposure of Terrestrial Vertebrate NTOs to Cry1B.868 and Cry1Da_7 in MON 95379 Maize

NTO Group	Test Species	Dietary Class	Body Weight (g)	FIR[a] (g diet/day)	Cry1B.868 EEC (mg/kg b.w.)	Cry1_Da7 EEC (mg/kg b.w.)
Wild Birds	*Colinus virginianus*	Herbivores	20	22.8	126.9	23.8
			100	64.9	72.4	13.6
			1000	290.8	32.4	6.1
		Granivores	20	5.1	10.4	0.1
			100	14.4	6.0	0.1
			1000	64.6	2.7	<0.1
Wild Mammals	*Mus musculus*	Herbivores	15	14.3	106.2	19.9
			35	23.1	73.4	13.8
			1000	152.8	17.0	3.2
		Granivores	15	3.2	8.7	0.1
			35	5.1	6.0	0.1
			1000	34.0	1.4	<0.1

[a]FIR = Food Intake Rate is the calculated dietary intake for each dietary and body weight class of birds and mammals (U.S. EPA T-REX v.1.5.2) based on U.S. EPA (1993).

[b]To calculate dose-based EECs, FIR values were multiplied by the 95th percentile protein expression in leaf for herbivores and grain for granivores and then divided by the nominal body weight values for each dietary and size class of birds and mammals.

Aquatic Organisms

Exposure of organisms in aquatic environments to Cry proteins through erosion of soil containing bound protein, surface runoff containing soluble protein, aerial deposition of pollen, and aerial deposition of crop dust can largely be ruled out (Carstens et al., 2012). Corn tissue can enter the aquatic environment immediately after harvest, but peak input typically occurs weeks or months later (Jensen et al., 2010). Exposure to aquatic organisms is predicted to be very low, since concentrations of Cry proteins in late-season crop tissues are typically less than what is measured during vegetative growth stages (Nguyen and Jehle, 2007).

Plant tissues must be conditioned in the aquatic environment for a period of approximately two weeks before it is utilized by aquatic organisms, considered to be "shredders", as a food source (Jensen et al., 2010). During this period of conditioning, soluble organic molecules rapidly leach from the tissue and the tissue surface is colonized by resident microorganisms (Carstens et al., 2012; Webster and Benfield, 1986). Using a sensitive insect bioassay, Jensen et al. (2010) did not detect Cry protein biological activity in senescent maize tissue two weeks after entering the aquatic environment. Cry3Bb1 was shown to dissipate rapidly from maize tissue in sediment microcosms, displaying a half-life within tissue of less than 3 days and was not detectable in the water or sediment phases over the course of the 30-day study (Prihoda and Coats, 2008). Cry1Ab has been shown to dissipate rapidly from maize tissue in aquatic microcosms with an estimated half-life of about two hours (Griffiths et al., 2017; Strain and Lydy, 2015). Recent work has shown that DvSnf7 RNA dissipates rapidly from maize tissue in aquatic microcosms with comparable kinetics to *Bt* proteins (Fischer et al., 2020). This suggests that the conclusion of rapid dissipation from maize tissue in aquatic environments can be generally applied to PIPs. It is therefore likely that the senescent maize tissue in an aquatic environment no longer retains Cry protein activity by the time it has been appropriately conditioned to be consumed by an aquatic shredder. Furthermore, these proteins are unlikely to persist or accumulate in aquatic environments resulting in minimal exposure to aquatic organisms.

Given the proposed growing areas of MON 95379 maize is limited to a maximum combined 100 acres per growing season, exposure to freshwater, estuarine, and marine environments is expected to be minimal. The reported rapid dissipation of Cry proteins in aquatic environments further supports the conclusion that expression of Cry1B.868 and Cry1Da_7 in MON 97539 maize poses minimal risk to aquatic organisms. Since exposure of MON 95379 maize to aquatic organisms is expected to be minimal, the risk to aquatic NTOs was not considered further in this assessment.

VII.C. Hazard Assessment

Tier-Based Approach for NTO Testing

Testing for potential effects of Cry1B.868 and Cry1Da_7 expressed in MON 95379 maize on non-target organisms (NTOs) was done according to U.S. regulatory guidelines for NTO testing and risk assessment of insect-protected crops (i.e., crops expressing PIPs). These guidelines were developed by USDA and EPA and suggest that testing and assessment be conducted based on a tier-based system (U.S. EPA, 2007). In this tiered approach, risk (a function of hazard and exposure) is evaluated within different levels or "tiers". Testing progresses from the lower tiers reflecting worst-case scenarios to increasingly more realistic exposure scenarios if the lower-tiered tests fail to indicate adequate certainty of acceptable risk. Additionally, the EPA has convened several Scientific Advisory Panel meetings to gather recommendations and provide guidance for NTO testing and risk assessment for PIPs (U.S. EPA, 2001b; c). Following the tiered approach, the Tier I study represents a worst-case exposure scenario to estimate hazard using an exposure pathway that is usually not realistic due to the high level of continuous exposure (e.g. no-choice laboratory test designs using artificial diets). Typically, an exposure factor of 10 times, a highly conservative exposure estimate, is administered in a Tier I test. EPA and USDA have established that "an endpoint of 50% mortality to be used as a trigger for additional higher tier testing" (U.S. EPA, 2007). Less than 50% mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic field exposure scenarios (U.S. EPA, 2007; 2010b; a). However, if an adverse effect (i.e. greater than 50% mortality) is observed under these conditions, this does not necessarily indicate the PIP poses an unacceptable risk in the field, but it does trigger the need for a better understanding of potential hazards at lower test concentrations using more realistic exposure conditions reflective of field concentrations (e.g. 1× the amount of the PIP expected to be available to the NTO under field conditions).

Test organisms were selected based on characteristics of the crop (maize) and the trait (insect protection). Based on the results from the activity spectrum assessment (Section VII.A) and known expression levels of the Cry1B.868 and Cry1Da_7 proteins in MON 95379 maize (Section VII.B), an evaluation of the potential toxicity to NTOs was conducted. Surrogate beneficial species used in Tier I laboratory tests were selected based on the results of the activity spectrum assessment and to ensure representation of different taxonomic groups, habitats and functions in the agro-ecosystem (maize fields), the characteristics of the crop (maize), the trait (insect protection) and the availability of robust Tier I test methods. The lack of adverse effects to surrogate NTOs representing an ecosystem function indicates that the ecosystem function will be maintained in the maize agro-ecosystem (U.S. EPA, 2007). The use of surrogate species in an NTO assessment is also in line with the strategy for tier-based testing for the effects of PIPs on non-target invertebrates described by the USDA and EPA (U.S. EPA, 2007), in which it was stated that "it is impossible to test all species that are potentially present…".

Tier I testing to assess the hazard of MON 95379 maize included laboratory toxicity testing against a representative pollinator [honey bee larvae and adults (*Apis mellifera*)], six beneficial insect species that represent biocontrol species [parasitic wasp (*Pediobius foveolatus*), ladybird beetle (*Colleomegilla maculata*), carabid beetle, (*Poecilus cupreus*), green lacewing (*Chrysoperla carnea and Chrysoperla rufilabris*), big-eyed bug (*Geocoris punctipes*)], and two representative soil taxa [earthworm (*Eisenia andrei*) and Collembola (*Folsomia candida*)].

The Tier I NTO studies performed with Cry1B.868 and Cry1Da_7 proteins followed methods based on either established EPA/OECD test guidelines or Bayer protocols that were sufficiently powered to detect adverse effects and evaluated biologically relevant endpoints. The Tier I assessment consisted of laboratory testing using intended exposure levels at least 10 times above conservative EEC values relevant for a given NTO species (Table VII-4). Ecologically relevant endpoints of survival and/or growth and development observations were assessed and tests were of sufficient duration to detect adverse effects based on the mode of action of the Cry1B.868 and Cry1Da_7 proteins. Acute studies were conducted with vertebrate NTOs as Cry proteins exhibit an acutely toxic MOA in sensitive species and there is low potential for chronic effects in vertebrate organisms (Koch et al., 2015). All studies met specific acceptance criteria to ensure their validity. All studies were conducted using purified Cry1B.868 and Cry1Da_7 proteins produced in a *Bt* expression system. These purified Cry1B.868 and Cry1Da_7 proteins were shown to be equivalent to the Cry1B.868 and Cry1Da_7 proteins expressed in MON 95379 maize based on apparent molecular weight, immunoreactivity, functional activity, and glycosylation, thus making them suitable surrogates to the plant-produced proteins for NTO testing.

The Cry1B.868 and Cry1Da_7 proteins have been shown to bind to different receptors and therefore act independently in lepidopteran insect pests (Wang et al., 2019). Laboratory bioassays with the sensitive fall armyworm species (*Spodoptera frugiperda*) demonstrated that the combined activity of Cry1B.868 and Cry1Da_7 is additive, further demonstrating these proteins act independently (Appendix J). When two or more proteins act independently and environmental concentrations of the proteins are below their no-effect levels, then the proteins can be assessed individually (U.S. EPA, 2009b; a); reviewed in Koch et al. (2015). Since Cry1B.868 and Cry1Da_7 have been shown to act independently when provided in combination to a sensitive species (Appendix J; (Wang et al., 2019)) interaction in NTOs at concentrations expected to be encountered in the field is not anticipated (Levine and Borgert, 2018; Levine et al., 2019). Furthermore, a supplementary analysis with a highly conservative hazard index approach indicates the additive activities of Cry1B.868 and Cry1Da_7 are below the levels of concern for NTOs (Appendix J). Therefore, Cry1B.868 and Cry1Da_7 proteins were assessed individually for the NTO assessment.

With the exception of the acute earthworm study, all studies on invertebrates utilizing the purified proteins included a diet analysis using a sensitive insect (FAW) bioassay to confirm that the Cry1B.868 and Cry1Da_7 proteins incorporated in the test diet were biologically active and had the expected level of biological activity. Additionally, where appropriate based upon the diet matrix, the homogeneity and stability of the Cry proteins in diet under test conditions and frozen storage was also confirmed. A dose confirmation is typically not necessary for the earthworm study because the test design is an acute study with a single application of test protein as opposed to continuous dietary exposure provided in other invertebrate Tier I tests. Dose confirmations in earthworm studies are also often challenging due to poor recovery from the artificial soil as well as temporal decline in soil concentrations commonly exhibited by Cry proteins (Ahmad et al., 2005; Dubelman et al., 2005; Head et al., 2002); reviewed in Icoz and Stotzky (2008). Details on study methods and results for all NTO tests are provided in Appendix J.

To characterize risk of Cry1B.868 and Cry1Da_7 to NTOs, a deterministic approach was used whereby MOEs were defined as the ratio of the *median lethal concentration* (LC_{50}) values to the EECs. Calculated MOEs were based on survival endpoints as mortality has been identified as the

primary endpoint in Tier I testing (U.S. EPA, 2007; 2010b). When necessary, sublethal endpoints were put into context of the ERA by comparing *no observed adverse effect concentration* (NOAEC) values to the EECs. Median lethal concentration and NOAEC values were derived from study results that are summarized in Appendix J. An EEC that is lower than the level required to cause an adverse effect is generally indicative of negligible risk and U.S. regulatory guidance states that only adverse effects to NTOs at field exposure levels (1×) are viewed as an environmental risk (U.S. EPA, 2007; 2010b; a). Since the study results from Tier I tests indicated no adverse effects at field exposure levels, higher tier studies were not conducted (U.S. EPA, 1998b; 2007).

VII.C.1. Effects of Cry1B.868 on NTOs

Predator and Parasitoids

Six species representing four different insect orders of predators and parasitoids were evaluated in Tier I toxicity tests where Cry1B.868 protein was provided under continuous dietary exposure. These species included *Coleomegilla maculata* (Coleoptera)*, Poecilus cupreus* (Coleoptera), *Geocoris punctipes* (Hemiptera), *Chrysoperla carnea and Chrysoperla rufilabris* (Neuroptera), and *Pediobius foveolatus* (Hymenoptera). For *C. maculata, P. cupreus, G. punctipes, and P. foveolatus,* no effects on survival were observed at all treatment levels including the highest concentration tested (3500 µg/g). The MOEs for these species derived from LC_{50} values and conservative EECs based on leaf expression was determined to be >31 indicating minimal risk to these tested predators and parasitoids (Table VII-8).

While no adverse effects were observed in a Tier I test with adult *G. punctipes* at 3500 µg/g, a statistically significant increase in development time (<1d) was observed at the lower 700 µg/g treatment level compared to the control (Appendix J). The magnitude of treatment-related effects based on the MOA of Cry proteins should be proportional to the test concentration. Since there was no dose-response evident between the treatment levels with no effects on development observed at a 5-fold higher concentration, it was determined that the less than 1 day effect on development time at the lower 700 µg/g test concentration was not treatment-related.

For *C. maculata* (ladybird beetle) a statistically significant decrease in adult body weight (<10%) at test completion was observed at 3500 µg/g, but not 700 µg/g when compared to the control group (Appendix J). An MOE for growth and development of *C. maculata* derived from a conservative EEC based on leaf expression and a NOAEC of 700 µg/g was determined to be ≥6. This is a highly conservative margin of exposure because the EC_{50} for adult body weight was >3500 µg/g. When prey is scarce, ladybird beetles may feed on pollen as a supplemental nutrition source and can constitute a significant portion of their diet (Berkvens et al., 2008; Duan et al., 2002). An MOE for growth and development based on exposure through pollen and a NOAEC of 700 µg/g was determined to be ≥11 indicating that Cry1B.868 is unlikely to have adverse sublethal effects on ladybird beetles at field-exposure levels.

Initial Tier I testing with adult *C. carnea* green lacewings resulted in statistically significant increased mortality following continuous dietary exposure to Cry1B.868 (Appendix J). When correcting for 15% control mortality, corrected mortality was 23.5% at the 3500 µg/g treatment level and 29.4% at the 700 µg/g. While statistically significant compared to the control, there was

no dose-response in mortality between the treatment levels and the estimated LC_{50} for *C. carnea* adults was >3500 µg/g and a NOAEC could not be determined.

In the absence of an observable dose-response, it is unclear whether the observed effects on survival in *C. carnea* were due to Cry1B.868 activity. Activity spectrum screening demonstrated that Cry1B.868 activity was limited to Lepidoptera and previous work with Cry1-family proteins have shown these proteins do not have adverse effects on green lacewing (reviewed in Romeis et al. (2014); Appendix L). It should be noted that the only ecologically relevant route of exposure for adult green lacewings is through consumption of pollen (Li et al., 2008; Li et al., 2010), as only the larval stages are predatory. The observed effects in this study occurred at concentrations well above those expected to be found in pollen (>11×; Table VII-4). These results indicate that it is unlikely that the mortality that was observed was due to toxicity from the Cry1B.868 protein. While guidance indicates that <50% effect at 10× a conservative EEC in Tier I testing is unlikely to result in risk under field conditions (U.S. EPA, 2007), without a finite LC_{50} or NOAEC defined, additional studies were conducted with lacewing larvae and adults to more fully evaluate potential effects of Cry1B.868 protein on green lacewings. Given delays in study initiation encountered with *C. carnea* due to challenges in getting sufficient numbers of quality insects and that species-level differences in lacewing sensitivity to Cry proteins is not anticipated, the additional larval and adult green lacewing studies were conducted with *C. rufilabris*; a species highly relavent to North American agriculture and commonly used in laboratory testing. A summary of the green lacewing studies and characterization of risk based on the results of these studies is described below.

A relevant route of exposure to green lacewing is consumption of herbivore prey that has fed on MON 95379 by green lacewing larvae, and larvae generally represent a more sensitive life-stage compared to adults (U.S. EPA, 2007). Therefore, to more fully characterize the potential effects of Cry1B.868 on green lacewing, a Tier I test was conducted with *C. rufilabris* larvae (Appendix J). *C. rufilabris* larvae were fed continuously on an artificial diet with Cry1B.868 protein incorporated at three treatment levels including 3500, 700, and 110 µg/g and were evaluated for pre-imaginal survival, development time to adult emergence, and weight at emergence. No effects on survival and growth and development of juvenile lacewings as well as emerged adult body weights were observed at all treatment levels including the highest concentration tested (3500 µg/g). The lack of effects on the lethal and sublethal endpoints at all concentrations tested, including those that far exceed the EEC, indicate that Cry1B.868 poses minimal risk to green lacewing larvae. The MOE for predatory *C. rufilabris* larvae, derived from a conservative EEC value based on leaf expression was determined to be >31 indicating minimal risk to green lacewing larvae (Table VII-8).

To further evaluate potential effects of Cry1B.868 on green lacewing adults, an additional Tier I study with *C. rufilabris* adult green lacewings was conducted. Newly emerged *C. rufilabris* adult green lacewings were fed continuously an artificial diet with Cry1B.868 incorporated at three treatment levels including 700, 350, and 110 µg/g and were evaluated for survival (Appendix J). The lower concentrations in the *C. rufilabris* adult test compared to the *C. carnea* test were chosen to capture a range of exposure levels more closely reflective of those expected to be found from exposure through pollen. The test design was more conservative than the *C. carnea* adult study described above in that the test diet was provided to organisms in a no-choice environment. No effects were observed on survival of *C. rufilabris* adults at any of the treatment levels including

the highest concentration tested (700 µg/g; Appendix L). Given that green lacewing adults would be exposed to Cry1B.868 solely through consumption of pollen, the MOE derived from an EEC value based on pollen expression is >11 indicating that Cry1B.868 as expressed in pollen poses minimal risk to green lacewing adults (Table VII-8).

The testing conducted with *C. rufilabris* covered the entire lacewing lifespan, included multiple lethal and sublethal endpoints, and evaluated multiple treatment levels including concentrations comparable to, as well as, far-exceeding those expected to be encountered in the field. Furthermore, exposure of lacewing larvae to Cry1B.868 is expected to be minimal as their preferred aphid prey have been shown to not efficiently take up Cry proteins expressed in maize (Romeis and Meissle, 2011); reviewed in Romeis et al. (2014). The temporally limited availability of maize pollen also minimizes exposure to green lacewing adults (Abendroth et al., 2011). In addition to the limited acreage under the proposed registration, the results of these analyses indicate that Cry1B.868 as expressed in MON 95379 maize poses minimal risk to green lacewing populations.

Table VII-8. Estimated Environmental Concentrations (EECs), Median Lethal Concentrations (LC_{50}) from Non-target Terrestrial Invertebrate Studies and Margins of Exposure (MOEs) for the Cry1B.868 Protein

Test Organism	Common Name	Order	EEC	LC_{50}	MOE
Coleomegilla maculata	Ladybird beetle	Coleoptera	111.4 µg/g leaf	>3500 µg/g	>31
Poecilus cupreus	Carabid beetle	Coleoptera	111.4 µg/g leaf	>3500 µg/g	>31
Geocoris punctipes	Big-eyed bug	Hemiptera	111.4 µg/g leaf	>3500 µg/ml	>31
Pediobius foveolatus	Parasitic wasp	Hymenoptera	111.4 µg/g leaf	>3500 µg/g	>31
Chrysoperla rufilabris (larva)	Green lacewing	Neuroptera	111.4 µg/g leaf	>3500 µg/g	>31
Chrysoperla rufilabris (adult)	Green lacewing	Neuroptera	61.9 µg/g pollen	>700 µg/g	>11
Folsomia candida	Springtail	Collembola	0.4 µg/g soil	>3500 µg/g	>8000
Eisenia andrei	Earthworm	Haplotaxida	0.4 µg/g soil	>3500 mg/kg soil	>8000
Apis mellifera (adult)	Honey bee	Hymenoptera	61.9 µg/g pollen	>900 µg/g	>14
Apis mellifera (larva)	Honey bee	Hymenoptera	61.9 µg/g pollen	>900 µg/g	>14

Pollinators

To test for potential effects of Cry1B.868 on pollinators, honey bee adults and larva (*Apis mellifera*) were tested in chronic Tier I toxicity tests. Cry1B.868 was provided continuously in diet for 10d to honey bee adults and no adverse effects were observed at any treatment level including the highest concentration tested (900 µg/g). To evaluate potential effects on honey bee larva, Cry1B.868 protein was provided in diet in a chronic honeybee larval test. No adverse effects were observed at any treatment level including the highest concentration tested (900 µg/g). Using a conservative EEC based on the expression of Cry1B.868 in pollen, the MOEs for adult and larval honeybees were determined to be >14 indicating minimal risk of Cry1B.868 to pollinators (Table VII-8).

Soil Organisms

Potential effects on soil organisms were evaluated in Tier I tests with earthworm (*Eisenia andrei*) and Collembola (*Folsomia candida*). No adverse effects were observed in earthworms and Collembola when Cry1B.868 was provided at 3500 µg/g in soil and diet, respectively. The MOEs were calculated by comparing the LC_{50} values to the EEC derived from the PEC of Cry1B.868 in soil. The MOEs for earthworm and Collembola were >8000 indicating minimal risk to soil organisms (Table VII-8).

Terrestrial Vertebrates

An avian acute oral toxicity test was conducted to test the effects of Cry1B.868 on avian species. Lyophilized Cry1B.868 protein was provided to quail at a nominal dose of 3500 mg protein/kg body weight. The LC_{50} of Cry1B.868 in this study was determined to be >3500 mg/kg. Using the smallest body weight as a worst-case dose-based EEC (Table VII-7), the MOEs for Cry1B.868 in wild bird species were determined to be >27 for herbivores and >336 for granivores (Table VII-9). The results from an acute oral toxicity test with mice were used to characterize risk to wild mammal populations. The LC_{50} for Cry1B.868 protein in mice was determined to be >5000 mg protein/kg body weight. Using the smallest body weight as a worst-case dose-based EEC for wild mammals (Table VII-7), the MOEs for Cry1B.868 were determined to be >47 for herbivores and >574 for granivores (Table VII-9). The estimated exposure level of Cry1B.868 to wild bird and mammal species is far below the highest levels tested where no adverse effects on survival were observed. These results support the conclusion that dietary exposure of Cry1B.868 poses negligible risk to wild bird and small mammals.

Table VII-9. Dose-Based Estimated Environmental Concentrations (EECs), Median Lethal Concentrations (LC50) from Terrestrial Vertebrate Studies and Margins of Exposure (MOEs) for the Cry1B.868 Protein

Test Organism (NTO group)	Dietary Class	EEC[a]	LC_{50}	MOE
Colinus virginianus (birds)	Herbivore	126.9 mg/kg b.w.	>3500 mg/kg b.w.	>27
	Granivore	10.4 mg/kg b.w.	>3500 mg/kg b.w.	>336
Mus musculus (mammals)	Herbivore	106.2 mg/kg b.w.	>5000 mg/kg b.w.	>47
	Granivore	8.7 mg/kg b.w.	>5000 mg/kg b.w.	>574

[a]The EEC value for the smallest body-weight vertebrate (Table VII-7) was used as a worst-case for risk characterization.

VII.C.2. Effects of Cry1Da_7 on NTOs

Predators and Parasitoids

Six species representing four different insect orders were evaluated in Tier I toxicity tests where Cry1Da_7 protein was provided under continuous dietary exposure. These species included *Coleomegilla maculata* (Coleoptera), *Poecilus cupreus* (Coleoptera), *Geocoris punctipes* (Hemiptera), *Chrysoperla carnea* and *Chrysoperla rufilabris* (Neuroptera), and *Pediobius foveolatus* (Hymenoptera). No adverse effects on survival were observed in all predators and parasitoids tested at the highest treatment level in provided diets (500 µg/g for all species with exception of 400 µg/g for *P. cupreus* and 150 µg/g corrected concentration for *C. maculata*). The MOEs for these species derived from LC50 values and conservative EECs based on leaf expression was determined to be >7. Consistent with EPA and USDA guidance, MOE values >1 support the conclusion of minimal risk to these tested predators and parasitoids (Table VII-8; (U.S. EPA, 2007)).

An increase in development time (<1 day) was observed in *C. maculata* (ladybird beetle) at both treatment levels (Appendix J). While statistically significant, such a small effect in development time is not anticipated to result in adverse effects in ladybird beetle populations under field conditions. EPA and USDA guidance states that <50% effect at 10× the EEC in a Tier I test is indicative of minimal risk (U.S. EPA, 2007). While it is not straight-forward to interpret a percent effect with endpoints such as development time, the magnitude of effect at the highest treatment concentration was 0.8d; a duration representing <4% of the total development time in the buffer control group with no apparent dose response between the two treatment levels. Additionally, there were no significant effects at any treatment level for the other endpoints in the study including survival and body weight of emerged adults. Lastly, the observed development times for the Cry1Da_7 treatments were less than those of the control groups in a companion study conducted with Cry1B.868. In other words, the difference in development time of the control groups between

the two studies was larger than the observed effect with Cry1Da_7. Therefore, the weight-of-evidence indicates that the effect observed in the development time endpoint will not adversely affect ladybird beetle populations.

While no adverse effects were observed in a Tier I test with adult *C. carnea* (green lacewing) at 500 µg/g, a significant increase in mortality was observed at the lower 50 µg/g treatment level (27 µg/g measured concentration) compared to the control. Since there was no dose-response evident between the treatment levels with no effects on survival observed at a >10-fold higher concentration, it was determined that the effects on survival at 27 µg/g were not treatment-related.

To fully confirm that Cry1Da_7 will not have adverse effects on green lacewing, the effects of Cry1Da_7 were evaluated on *C. rufilabris* larvae, which represent a potentially more sensitive and ecologically relevant growth stage (Appendix J). Cry1Da_7 was incorporated into an artificial diet at 500 and 50 µg/g diet and fed continuously to *C. rufilabris* larva. Effects on pre-imaginal survival, development time to adult emergence, and weight at emergence were evaluated. Following continuous dietary exposure of Cry1Da_7 protein, no adverse effects were observed on survival, growth and development of *C. rufilabris* larvae. The results of these tests indicate that Cry1Da_7 will not have adverse effects on green lacewing.

When the LC_{50} values from Tier I tests with species representing predators and parasitoids were compared to conservative EEC values, the MOEs were determined to be >19 with the exception of *C. maculata*, where the MOE was >7 (Table VII-10). The MOE for *C. maculata* was relatively lower compared to other tested predators and parasitoids because of a correction for measured activity in test diets and not due to an observed adverse effect. Although leaf expression values were conservatively used to calculate EEC for *C. maculata*, it should be noted that the preferred prey for ladybird beetles are aphids (Tian et al., 2015). Exposure of *C. maculata* to Cry1Da_7 through aphid prey is expected to be minimal as aphids have been shown to not efficiently take up Cry proteins expressed in maize (Romeis and Meissle, 2011; Tian et al., 2015); reviewed in Romeis et al. (2014). The other potential route of exposure is consumption of pollen as a supplemental nutrition source and the MOE for *C. maculata* based on pollen exposure is >1500 as Cry1Da_7 is not detectable in pollen. Therefore, the results of these Tier I studies and risk characterization indicate that adverse effects from Cry1Da_7 as expressed in MON 95379 maize on beneficial predators and parasitoids is not anticipated.

Table VII-10. Estimated Environmental Concentrations (EECs), Median Lethal Concentrations (LC_{50}), from Non-target Terrestrial Invertebrate Studies and Margins of Exposure (MOEs) for the Cry1Da_7 Protein

Test Organism	Common Name	Order	EEC	LC_{50}	MOE
Coleomegilla maculata	Ladybird beetle	Coleoptera	20.9 µg/g leaf	>150 µg/g[a]	>7
Poecilus cupreus	Carabid beetle	Coleoptera	20.9 µg/g leaf	>400 µg/g	>19
Geocoris punctipes	Big-eyed bug	Hemiptera	20.9 µg/g leaf	>500 µg/ml	>23
Pediobius foveolatus	Parasitic wasp	Hymenoptera	20.9 µg/g leaf	>500 µg/g	>23
Chrysoperla rufilabris (larva)	Green lacewing	Neuroptera	20.9 µg/g leaf	>500 µg/g	>23
Chrysoperla carnea (adult)	Green lacewing	Neuroptera	0.1 µg/g pollen	>500 µg/g	>5000
Folsomia candida	Springtail	Collembola	0.2 µg/g soil	>500 µg/g	>2500
Eisenia andrei	Earthworm	Haplotaxida	0.2 µg/g soil	>500 mg/kg soil	>2500
Apis mellifera (adult)	Honey bee	Hymenoptera	0.1 µg/g pollen	>500 µg/g	>5000
Apis mellifera (larva)	Honey bee	Hymenoptera	0.1 µg/g pollen	>500 µg/g	>5000

[a]No adverse effects on survival observed at nominal concentration of 415 µg/g. The nominal concentration for Cry1Da_7 was corrected for measured activity in test diet.

Pollinators

To evaluate potential effects of Cry1Da_7 on pollinators, honey bee adults and larvae (*Apis mellifera*) were tested in chronic Tier I toxicity tests. Cry1Da_7 was provided continuously in diet for 10d to honey bee adults and no adverse effects were observed at any treatment level including the highest concentration tested (500 µg/g). To evaluate potential effects on honey bee larvae, Cry1Da_7 protein was provided in diet in a chronic honeybee larval test. No adverse effects were observed at any treatment level, including the highest concentration tested (500 µg/g). Since the concentration of Cry1Da_7 in pollen is below the limits of quantitation for the ELISA assay, a value equivalent to the method LOQ was used as a conservative estimate of exposure. The MOEs for adult and larval honeybees were >5000 indicating minimal risk of Cry1Da_7 to pollinators (Table VII-10).

Soil Organisms

Potential effects on soil organisms were evaluated in Tier I tests with earthworm (*Eisenia andrei*) and Collembola (*Folsomia candida*). No adverse effects were observed in earthworms and Collembola when Cry1Da_7 was provided at 500 µg/g in soil and diet, respectively. The MOEs were calculated comparing the LC_{50} values to the EEC derived from the PEC for Cry1Da_7 in soil. The MOEs for both species were >2500 indicating minimal risk to soil organisms (Table VII-10).

Terrestrial Vertebrates

An avian acute oral toxicity test was conducted to test the effects of Cry1Da_7 on avian species. Lyophilized Cry1Da_7 protein was provided to quail at a nominal dose of 1000 mg protein/kg body weight. No adverse effects were observed over the course of the study and the LC_{50} of Cry1Da_7 was determined to be >1000 mg/kg body weight. Using the smallest body weight as a worst-case dose-based EEC (Table VII-7), the MOEs for Cry1Da_7 in wild birds were determined to be >42 for herbivores and >10,000 for granivores (Table VII-11). The results from an acute oral toxicity test with mice were used to characterize risk to wild mammal populations. The LC_{50} of Cry1Da_7 protein was determined to be >5000 mg protein/kg body weight. Using the smallest body weight as a worst-case dose-based EEC for wild mammals (Table VII-7), the MOEs for Cry1Da_7 were determined to be >250 for herbivores and >50,000 for granivores (Table VII-11). The estimated exposure level of Cry1Da_7 to wild bird and mammal species is far below the highest levels tested where no adverse effects were observed. These results support the conclusion that dietary exposure of Cry1Da_7 poses negligible risk to birds and small mammals.

Table VII-11. Dose-Based Estimated Environmental Concentrations (EECs), Median Lethal Concentrations (LC_{50}) from Terrestrial Vertebrate Studies and Margins of Exposure (MOEs) for the Cry1Da_7 Protein

Test Organism (NTO group)	Dietary Class	EEC[a]	LC_{50}	MOE
Colinus virginianus (birds)	Herbivore	23.8 mg/kg b.w.	>1000 mg/kg b.w.	>42
	Granivore	0.1 mg/kg b.w.	>1000 mg/kg b.w.	>10,000
Mus musculus (mammals)	Herbivore	19.9 mg/kg b.w.	>5000 mg/kg b.w.	>250
	Granivore	0.1 mg/kg b.w.	>5000 mg/kg b.w.	>50,000

[a]The EEC value for the smallest body-weight vertebrate (Table VII-7) was used as a worst case for risk characterization.

VII.D. Effect of MON 95379 Maize on Threatened and Endangered Species

A comprehensive assessment on the potential effects of MON 95379 maize on listed threatened and endangered species was conducted. While MON 95379 will not be commercialized in the U.S., limited breeding and seed increase related activities are intended to occur in Nebraska, Hawaii, and Iowa on up to a combined maximum U.S. total of 100 acres per growing season and small-scale, confined field trials may potentially occur in other locations to collect needed regulatory data to support global regulatory submissions of future products stacked with MON 95379. Since the results of activity spectrum screening and Tier I testing indicated effects on survival from Cry1B.868 and Cry1Da_7 are limited to Lepidoptera, a comparison of the county level distribution of endangered lepidopteran species in these three states relative to maize production counties identified three species for further evaluation (Table VII-12). These species include Blackburn's sphinx moth (*Manduca blackburni*) in Hawaii, Dakota skipper (*Hesperia dacotae*) and the Poweshiek skipperling (*Oarisma Poweshiek*) in Iowa and no species in Nebraska. Evaluation of critical habitats and updated ranges for these species supports the conclusion that Blackburn's sphinx moth, Dakota skipper, and Poweshiek skipperling would have no significant exposure to MON 95379 maize. In support of our assessment on likely exposure, a recent assessment of potential effects on threatened and endangered species was conducted by U.S. EPA for MON 89034 × TC1507 × MON 87411 × DAS-59122-7 maize, that expresses lepidopteran-active Cry1A.105, Cry2Ab2, and Cry1F proteins, resulted in a No Effect determination for listed lepidopteran species, including Blackburn's sphinx moth, due to habitat requirements that exclude them from maize fields (U.S. EPA, 2017). Recent surveys by U.S. FWS indicate that Dakota skipper and Poweshiek skipperling have not been observed in Iowa since the time of listing (2014) and are no longer believed to be present in Iowa (USFWS, 2018a; b). The specificity of Cry1B.868 and Cry1Da_7 for Lepidoptera and negligible expected exposure based on the proposed limited acreage and use of standard agronomic management practices for small scale field trials results in

the conclusion that MON 95379 will have no effect on currently listed threatened and endangered species (see also Appendix L.21).

Table VII-12. Spatial Overlap of Threatened and Endangered Species Habitat and Maize Cultivation in Nebraska, Hawaii, and Iowa

Common Name	Species Name	Reference	Habitat	Overlap of Species Presence with Maize-Production Counties[a]
Blackburn's sphinx moth	*Manduca blackburni*	USFWS, 2005	Mesic shrubland	Counties: 2 HI: Hawaii, Maui
Dakota skipper	*Hesperia dacotae*	USFWS, 2018a	Prairie grasslands	Counties: 1[b] IA: Dickinson
Poweshiek skipperling	*Oarisma powershiek*	USFWS, 2018b	Prairie grasslands	Counties: 7[c] IA: Cerro Gordo, Dickinson, Emmet, Hancock, Howard, Kossuth, Osceola

[a]County-level maize production estimates derived from USDA-NASS 2017 Agricultural Census (USDA-NASS, 2017).
[b]Recent update by USFWS indicate this species is no longer believed to be present in Iowa (USFWS, 2018a).
[c]Species has not been observed in Iowa since the time of listing and is no longer believed to be present in the United States outside of Michigan (USFWS, 2018b).

VII.E. Conclusion

MON 95379 maize expresses Cry1B.868 and Cry1Da_7 whose activity is limited to Lepidoptera under field conditions. Cry1B.868 and Cry1Da_7 are members of the Cry1 family of *Bt* proteins. Cry1B.868 and Cry1Da_7 have a MOA shared with previously commercialized Cry1 proteins that have a long history of safe use (Koch et al., 2015; Wang et al., 2019). Under the proposed terms of an EPA seed increase registration application, MON 95379 maize will be cultivated for small-scale breeding, testing, and seed increase-related activities in the three U.S. states of Nebraska, Hawaii, and Iowa with a maximum combined total acreage of 100 acres per growing season and small-scale, confined field trials potentially in other locations to collect needed regulatory data to support global regulatory submissions of future products stacked with MON 95379. This limited geography significantly limits exposure to NTOs beneficial to agriculture. Tier I toxicity testing on NTOs representing key ecological functions demonstrated no adverse effects on survival from Cry1B.868 and Cry1Da_7 at levels expressed in MON 95379 maize. Calculated MOEs based on conservative estimates of exposure were >11 for all species tested with the exception of *C. maculata* where the MOE for Cry1Da_7 was >7. These results indicate no adverse effects to NTOs are anticipated at field expression levels. Therefore, the results of the assessment based on the studies described herein support the conclusion that intended cultivation of MON 95379 maize under the proposed limited acreage poses minimal risk to NTO beneficial to agriculture. An evaluation focused on listed Lepidoptera species in the states of Nebraska, Hawaii, and Iowa, where MON 95379 will be primarily cultivated, resulted in the conclusion of no effect for currently listed threatened and endangered species.

VIII. PHENOTYPIC, AGRONOMIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

This section provides a comparative assessment of the phenotypic, agronomic, and environmental interactions characteristics of MON 95379 compared to the conventional control. The data support a conclusion that MON 95379 is unlikely to pose a greater plant pest risk than conventional maize. This conclusion is based on the results of multiple evaluations including field and laboratory assessments. MON 95379 has been planted in the U.S. under permit/notification since 2015 (Appendix A).

Phenotypic, agronomic, and environmental interactions characteristics of MON 95379 were evaluated in a comparative manner to assess plant pest potential. These assessments included seed germination and dormancy characteristics and as well as phenotypic and agronomic characteristics and plant responses to abiotic stressors, diseases, and arthropod pests in the field. Results from these assessments demonstrate that MON 95379 does not possess 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests; or 3) characteristics that would confer an increased plant pest risk compared to conventional maize.

VIII.A. Characteristics Measured for Assessment

In the phenotypic, agronomic, and environmental interactions assessment of MON 95379, data were collected to evaluate altered plant pest potential. A detailed description of the regulated article phenotype is requested as part of the petition for determination of nonregulated status in the previous regulations codified in 7 CFR § 340.6 including differences from the unmodified recipient organism that would "substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived." As part of the characterization of MON 95379, data were collected to provide a detailed description of the phenotypic, agronomic, and environmental interactions characteristics of MON 95379. A subset of these data represents specific characteristics that are typically associated with altered plant pest potential (e.g., seed dormancy, lodging, seed loss, and environmental interactions data).

The plant characterization of MON 95379 encompassed five general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development; 4) lodging and seed retention on the plant; and 5) plant responses to abiotic stressors, diseases, and arthropod pests. An overview of the characteristics assessed is presented in Table VIII-1.

The phenotypic, agronomic, and environmental interactions data were evaluated from a basis of familiarity (OECD, 1993) and were comprised of a combination of field and laboratory studies conducted by scientists who are familiar with the production and evaluation of maize. In each of these assessments, MON 95379 was compared to an appropriate conventional control that had a genetic background similar to MON 95379 but did not possess the lepidopteran-protected trait. In addition, multiple commercial maize hybrids developed through conventional breeding and selection (see Appendix G-Appendix I and Tables G-1, H-1, and I-1) were included to provide a range of comparative values for each characteristic that are representative of the variability in existing commercial maize hybrids. Data collected for the various characteristics from the commercial reference maize hybrids provide context for interpreting experimental results.

Table VIII-1. Phenotypic, Agronomic, and Environmental Interaction Characteristics Evaluated in U.S. Field Trials and Laboratory Studies

Data category	Characteristic measured (section where discussed)	Evaluation timing (setting of evaluation)	Evaluation description (measurement endpoints)
Germination, dormancy, and emergence	Germinated (VIII.C.1)	Day 4 and 7 at OT[1] Day 4, 7, and 11 at SOT[1] (Laboratory)	Percentage of seed with a radicle protruding through the seed coat and greater than 1 mm in length
	Dead (VIII.C.1)	Day 4 and 7 at OT Day 4, 7, and 11 at SOT (Laboratory)	Percentage of seed that had visibly deteriorated and become soft to the touch (also included non-viable hard and non-viable firm-swollen seed)
	Viable firm-swollen (VIII.C.1)	Day 7 at OT Day 11 at SOT (Laboratory)	Percentage of seed that imbibed water and were firm to the touch but lacked any evidence of growth (viability determined by a tetrazolium test)
	Viable hard (VIII.C.1)	Day 7 at OT Day 11 at SOT (Laboratory)	Percentage of seed that did not imbibe water and remained hard to the touch (viability determined by a tetrazolium test)
	Early stand count (VIII.C.2.1)	V2 – V4 growth stage (Field)	Number of plants per m^2
Vegetative growth	Plant height (VIII.C.2.1)	R1 – R6 (Field)	Distance from the soil level to the flag leaf collar
	Days to maturity (VIII.C.2.1)	R6 (Field)	Number of days from planting to kernel black layer
	Final stand count (VIII.C.2.1)	Pre-harvest (Field)	Number of plants per m^2

[1] Optimum temperature OT = 25°C for seven days, Suboptimum temperature SOT = 10°C for seven days followed by 25°C for four days.

Table VIII-I. Phenotypic, Agronomic, and Environmental Interactions Characteristics Evaluated in U.S. Field Trials and Laboratory Studies (continued)

Data category	Characteristic measured (section where discussed)	Evaluation timing (setting of evaluation)	Evaluation description (measurement endpoints)
Reproductive development	Days to flowering (VIII.C.2.1)	R1 (Field)	Days from planting until 50% of the plants have begun to shed pollen
	Grain moisture (VIII.C.2.1)	Harvest (Field)	Percentage moisture of harvested grain
	Seed weight (VIII.C.2.1)	Harvest (Field)	Mass of 100 mature seeds, adjusted to 15.5% moisture content
	Yield (VIII.C.2.1)	Harvest (Field)	Mass of harvested grain per hectare, adjusted to 15.5% moisture content
Lodging and seed retention	Lodging (VIII.C.2.1)	Pre-harvest (Field)	Percentage of plants leaning >45° from vertical or broken below the ear
	Seed loss (VIII.C.2.1)	Pre-harvest (Field)	Number of ears completely detached from plants in two rows
Environmental interactions	Abiotic stress response (VIII.C.2.2)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Disease damage (VIII.C.2.2)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Arthropod damage (VIII.C.2.2)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)

[1] Optimum temperature OT = 25°C for seven days, Suboptimum temperature SOT = 10°C for seven days followed by 25°C for four days.

VIII.B. Interpretation of Phenotypic and Environmental Interaction Data

Plant pest risk assessments for biotechnology-derived crops are comparative assessments and are considered from a basis of familiarity. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a well-characterized conventional maize whose biological properties and plant pest potential are well-known. Familiarity considers the biology of the crop, the introduced traits, the receiving environment and the interaction of these factors, and provides a basis for comparative environmental risk assessment between a biotechnology-derived plant and its conventional counterpart.

Expert knowledge and experience with conventionally bred maize was the basis for selecting appropriate endpoints and estimating the range of responses that would be considered typical for maize. As such, MON 95379 was compared to the conventional control in the assessment of phenotypic, agronomic, and environmental interactions characteristics. Based on all of the data collected, an assessment was made to determine if MON 95379 could be expected to pose an increased plant pest risk compared to conventional maize.

VIII.B.1. Interpretation of Phenotypic and Agronomic Data

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased weediness or plant pest risk. Under the framework of familiarity, characteristics for which no differences are detected support a conclusion of no increased weediness or plant pest risk of the biotechnology-derived crop compared to the conventional crop. Characteristics for which statistically significant differences are detected are considered in a step-wise method (Figure VIII-1) or in a similar fashion. All detected differences for a characteristic are considered in the context of whether or not the difference would increase the crop's pest/weed potential. Ultimately, a weight of evidence approach considering all characteristics and data is used for the overall risk assessment of differences and their significance. Figure VIII-1 illustrates the stepwise assessment process employed.

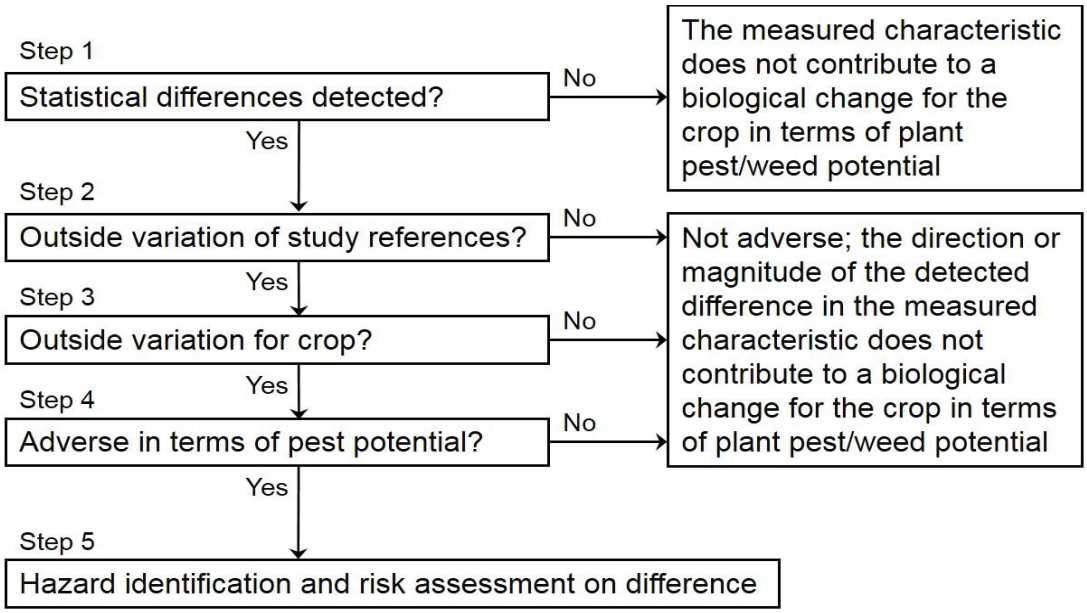

Figure VIII-1. Interpretation of Statistical Differences

Step 1 – Evaluate Detected Statistically Significant Differences

Data on each measured characteristic are statistically analyzed. A combined-site analysis is used for multi-site data. All statistically significant differences are evaluated and considered in the context of a change in weediness or plant pest risk. Any difference detected is further assessed.

Step 2 – Evaluate Differences in the Context of Commercial Reference Materials Included in the Study

If a difference for a characteristic is detected then the mean value of the biotechnology-derived crop for the characteristic is assessed relative to the range of variation of the commercial reference materials included in the study (e.g., reference range).

Step 3 – Evaluate Differences in the Context of the Crop

If the mean value of the characteristics for a biotechnology-derived crop is outside the variation of the commercial reference materials included in the study, the mean value of the biotechnology-derived crop is assessed relative to known values common for the crop (e.g., published values).

Step 4 – Relevance of Difference to Weediness or Plant Pest Risk

If the mean value of the characteristics for a biotechnology-derived crop is outside the range of values common for the crop, the difference is then assessed for whether or not it is meaningful in terms of weediness or plant pest risk.

Step 5 – Conduct Risk Assessment on Identified Hazard

If an adverse effect (hazard) is identified, a risk assessment on the difference is conducted. The risk assessment considers contributions to enhanced weediness or plant pest risk of the crop itself, the impact of differences detected in other measured characteristics, and potential for and effects of trait introgression into any populations growing outside of cultivated environments or into a sexually compatible species.

VIII.B.2. Interpretation of Environmental Interactions Data

The environmental interactions data consisting of plant responses to abiotic stressors, diseases, and arthropod pests are categorical and were considered to differ in susceptibility or tolerance if the range of injury symptoms did not overlap between the biotechnology-derived crop and the conventional control across all four replications within an observation at a site.

Observations for which no differences are observed support a conclusion of no increased weediness or plant pest risk. Observations for which differences were detected are not considered to indicate an increase in weediness or plant pest risk if the biotechnology-derived crop stressor responses and damage ratings are within the reference range or are not consistently observed in multiple environments in which the same stressor occurred.

VIII.C. Comparative Assessments of the Phenotypic, Agronomic, and Environmental Interaction Characteristics of MON 95379

This section provides the results of comparative assessments conducted in replicated laboratory and/or multi-site field experiments to provide a detailed phenotypic, agronomic, and environmental interactions description of MON 95379. The characteristics for MON 95379 evaluated in these assessments included: seed germination and dormancy characteristics (Section VIII.C.1), plant phenotypic, agronomic, and environmental interactions observations under field conditions (Section VIII.C.2). Additional details for each assessment are provided in Appendix G and Appendix H.

VIII.C.1. Seed Dormancy and Germination Characteristics

USDA-APHIS considers the potential for weediness to constitute a plant pest factor (previously 7 CFR § 340.6). Seed germination and dormancy mechanisms vary with species and their genetic basis tends to be complex. Seed dormancy (e.g., hard seed) is an important characteristic that is often associated with plants that are considered weeds (Anderson, 1996; Lingenfelter and Hartwig, 2007). Information on germination and dormancy characteristics is therefore useful when assessing a plant for increased weediness potential. To assess germination characteristics, standardized germination assays are available and routinely used. The Association of Official Seed Analysts (AOSA), an internationally recognized seed testing organization, recommends a temperature regime of 25°C for testing the germination and dormancy characteristics of maize seed (AOSA, 2018b; a). The AOSA further recognizes a temperature regime of constant 10°C for seven days followed by 25°C for four days for cold testing of maize (AOSA, 2009).

A comparative assessment of seed germination and dormancy characteristics was conducted for MON 95379 and the conventional control. The seed lots for MON 95379 and the conventional control were harvested from one 2018 field production site in Kihei, Hawaii. Four reference maize hybrids were obtained from commercial sources. MON 95379 was compared to the conventional control for percentages of germinated, dead, viable firm-swollen, and viable hard seed using two temperature regimes: optimum treatment was performed at 25ºC for 7 days; suboptimum treatment was performed at 10ºC for 7 days, followed by 4 days at 25ºC. The assay for each temperature regime was conducted using a randomized complete block design with four replications. Descriptions of the evaluated germination and dormancy characteristics and the timing of the

evaluations are listed in Table VIII-1. The materials and experimental methods are further discussed in Appendix G.

In the analyses of the dormancy and germination data, no statistically significant differences ($\alpha = 0.05$) were detected between MON 95379 and the conventional control in either the optimum (25°C) or suboptimum (10/25°C) temperature regimes for any of the evaluated characteristics, including viable hard seed (Table VIII-2). These results support the overall conclusion that the introduction of lepidopteran-protected trait does not result in increased weediness or plant pest risk of MON 95379 compared to conventional maize.

Table VIII-2. Germination Characteristics of MON 95379 and the Conventional Control

Temperature Treatment[2]	Characteristic[3]	Mean % (SE)[1]		Reference Range[4]
		Test	Control	
Optimum	Germinated	99.5 (0.50)	100.0 (0.00)	98.8 – 99.3
	Dead	0.5 (0.50)	0.0 (0.00)	0.5 - 1.3
	Viable Firm-Swollen	0.0 (0.00)	0.0 (0.00)	0.0 - 0.5
	Viable Hard	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
Suboptimum	Germinated	99.3 (0.48)	100.0 (0.00)	99.3 – 99.5
	Dead	0.0 (0.00)	0.0 (0.00)	0.0 - 0.5
	Viable Firm-Swollen	0.8 (0.48)	0.0 (0.00)	0.0 - 0.5
	Viable Hard	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0

Note: The experimental design was a randomized complete block with 4 replicates.
[1]Values represent means with standard error (SE) in parentheses.
[2]Optimum treatment was performed at 25ºC for 7 days; suboptimum treatment was performed at 10ºC for 7 days, followed by 4 days at 25ºC.
[3]Statistical comparisons were performed using Fisher's Exact Test. No statistical comparisons were made if test and control values were 0.
[4]Minimum and maximum mean values observed among the reference materials.

VIII.C.2. Field Phenotypic, Agronomic, and Environmental Interactions Characteristics

Phenotypic and agronomic characteristics were evaluated under field conditions as part of the plant characterization assessment of MON 95379. These data were developed to provide USDA-APHIS with a detailed description of MON 95379 relative to the conventional control and reference maize hybrids. According to previous regulations codified in 7 CFR § 340.6, as part of the petition to seek deregulation, a petitioner must submit "a detailed description of the phenotype of the regulated article." This information is being provided to assess whether there are phenotypic differences between MON 95379 and the conventional control that may impact its weediness or plant pest risk. Specific characteristics that are typically associated with weediness (e.g., lodging and seed loss) were used to assess whether there is a potential increase in weediness of MON 95379 compared to conventional maize.

USDA-APHIS considers the environmental interaction of the biotechnology-derived crop compared to its conventional control to determine the potential for increased plant pest

characteristics. Evaluations of environmental interactions were conducted as part of the plant characterization for MON 95379. In the 2018 U.S. field trials conducted to evaluate the phenotypic and agronomic characteristics of MON 95379, data were also collected on plant responses to abiotic stressors, diseases, and arthropod pests.

Data were collected from eight 2018 field sites within maize production regions of the U.S. (Table VIII-3). The test material MON 95379, the conventional control, and four commercial reference hybrids were planted at each site in a randomized complete block design with four replications. Seventeen unique references were included among the sites. Additional details on the materials and methods are presented in Appendix H.

VIII.C.2.1. Field Phenotypic and Agronomic Characteristics

MON 95379 was compared to the conventional control in a combined-site analysis for nine phenotypic and agronomic characteristics: early stand count, days to flowering, plant height, days to maturity, lodging, final stand count, moisture, seed weight, and yield. Descriptive statistics are provided for an additional characteristic, seed loss, that had insufficient variability for formal statistical analysis. Descriptions of the evaluated phenotypic and agronomic characteristics and the timing of the evaluations are listed in Table VIII-1. The materials and methods are further discussed in Appendix H.

The means for MON 95379 and the conventional control for seed loss, which was excluded from formal analysis, were low and comparable. The mean for MON 95379 was within the reference range. These results suggest that this characteristic does not contribute to a biological change for MON 95379 in terms of plant pest potential.

In the combined-site analysis of phenotypic characteristics of the MON 95379 compared to the conventional control, no statistically significant differences ($\alpha = 0.05$) were detected between the test and the conventional control for 7 of the 9 analyzed phenotypic characteristics. The MON 95379 test had significantly higher final stand and lower seed weight compared to the conventional control. However, the mean value(s) of MON 95379 were within respective reference ranges of conventional maize for these characteristics (Table VIII-4). These results, together with those for seed loss, support the overall conclusion that the introduction of the lepidopteran-protected trait does not result in increased weediness or plant pest risk of MON 95379 compared to conventional maize.

Table VIII-3. Phenotypic and Agronomic and Environmental Interactions Sites for MON 95379 during 2018

Site Code	County, State
IAEH	Shelby, Iowa
IAOG	Boone, Iowa
IARL	Jefferson, Iowa
ILHY	Clinton, Illinois
ILJO	Champaign, Illinois
INSH	Clinton, Indiana
NEYO	York, Nebraska
OHTR	Miami, Ohio

Table VIII-4. Combined-Site Analysis of Phenotypic and Agronomic Characteristics of MON 95379 Compared to the Conventional Control in 2018 U.S. Field Trials

Characteristic (units)	Mean (S.E.)[1]		Reference Range[2]
	MON 95379	Control	
Early stand count (plants/m^2)	8.6 (0.10)	8.5 (0.08)	7.6 – 9.4
Days to flowering	58.5 (0.62)	57.9 (0.60)	51.0 – 63.2
Plant height (cm)	263.0 (2.07)	257.2 (2.19)	225.3 – 267.0
Days to maturity	119.2 (1.34)	120.4 (1.36)	106.0 – 126.0
Lodging (%)	12.9 (2.37)	9.4 (1.68)	0.4 – 18.9
Seed loss (ears)[†]	0.0 (0.03)	0.0 (0.03)	0.0 – 0.5
Final stand count (plants/m^2)	8.2 (0.10)*	8.0 (0.10)	7.3 – 8.9
Moisture (%)	17.1 (0.24)	17.1 (0.22)	15.2 – 19.1
Seed weight (g)	34.1 (0.51)*	35.8 (0.48)	29.3 – 40.5
Yield (t/ha)	13.2 (0.34)	13.3 (0.35)	12.0 – 18.6

Notes: *Indicates a statistically significant difference between MON 95379 and the conventional control ($\alpha = 0.05$) in the combined-site, linear mixed model analysis. All plots at sites INSH and NEYO were thinned to promote uniform density following early stand count.

[†] Indicates that p values could not be generated because the data did not meet the normality and/or common variance assumptions of the linear mixed model.

[1] N = 32 for means. S.E. = standard error.

[2] Minimum and maximum mean values among 17 references, where each mean was combined over all the sites at which the reference hybrid was planted.

VIII.C.2.2. Environmental Interaction Assessments

Environmental interaction assessments evaluate the potential for unintended effects by seeking to determine whether the biotechnology derived plant demonstrates an increase in susceptibility or tolerance to abiotic stressors, diseases and arthropod pests. Plant responses to abiotic stressors, diseases, and arthropod pests were assessed at natural levels, i.e., no artificial infestation or imposed abiotic stress; therefore, these levels typically varied between observations at a site and among sites. These data were collected from each plot using a categorical scale (none, slight, moderate, and severe) of increasing severity of observed damage for each stressor. This scale was utilized to allow for the evaluation of the wide variety of potential abiotic stressors, diseases, and

arthropod pests occurring across the season and across sites. These data were summarized and not subjected to ANOVA statistical evaluation. For a particular stressor, all comparisons of the range of responses for MON 95379 to the range of responses for the conventional control across all observation times and sites are reported. Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VIII-1. The materials, methods, additional details concerning the qualitative environmental interactions assessments, and detailed results of the qualitative data comparisons are further discussed and presented in Appendix H and Appendix L.

In an assessment of plant responses to abiotic stressors, diseases, and arthropod pests, no differences were observed between MON 95379 and the conventional control for any of the 288 observations (including 96 abiotic stressor, 96 disease, and 96 arthropod pest observations) among the eight sites (Table VIII-5). These results support no increased susceptibility or tolerance to specific abiotic stressors, diseases or non-target arthropod pests of MON 95379 compared to conventional maize.

Table VIII-5. Summary of Qualitative Environmental Interactions Assessments for MON 95379 during 2018

Stressor	Number of observations across all sites	Number of observations with no differences between MON 95379 and the conventional control across all sites
Abiotic stressors	96	96
Diseases	96	96
Arthropod pests	96	96
Total	288	288

No differences were observed between MON 95379 and the conventional control during any of the 96 observations (8 sites, 4 timepoints, 3 stressors) for each stressor category. MON 95379 and the conventional control were considered different in susceptibility or tolerance if the range of injury symptoms across four replications did not overlap between MON 95379 and the conventional control.

VIII.D. Conclusions for Phenotypic, Agronomic, and Environmental Interactions Evaluation

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest potential as assessed by USDA-APHIS. Under the framework of familiarity, characteristics for which no statistically significant differences are detected support a conclusion of no increased plant pest potential of the biotechnology-derived crop compared to the conventional crop. Ultimately, a weight of evidence approach that considers all characteristics and data is used for the overall risk assessment of differences and their significance.

An extensive and robust set of phenotypic, agronomic, and environmental interactions data were used to assess whether the introduction of the lepidopteran-protected trait altered the plant pest potential of MON 95379 compared to the conventional control, considered within the context of the variation among the reference maize hybrids. These assessments included five general data

categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development; 4) lodging and seed retention on the plant; and 5) plant responses to abiotic stressors, diseases, and arthropod pests. Within these data categories, specific characteristics typically associated with weediness were also assessed to determine whether there was a potential increase in weediness of MON 95379 compared to conventional maize.

Results from these assessments comparing MON 95379 and the conventional control support the conclusion that MON 95379 does not possess: 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests; or 3) characteristics that would confer a plant pest risk compared to conventional maize. Therefore, based on the results of multiple assessments discussed above and presented in the appendices, the weight of evidence supports the overall conclusion that the introduction of the lepidopteran-protected trait does not result in increased weediness or plant pest risk of MON 95379 compared to conventional maize.

IX. U.S. AGRONOMIC PRACTICES

IX.A. Introduction

As part of the plant pest assessment required by the previous regulations codified in 7 CFR § 340.6(c)(4), impacts to agricultural and cultivation practices must be considered. MON 95379 will not be commercialized in the U.S. and therefore will not impact U.S. commercial maize agricultural practices. It is intended to be cultivated in small scale breeding, testing, and seed increase nurseries to develop seed for future products in South America. In the future, MON 95379 stack products may also be planted in confined, small-scale field trials that would support subsequent global regulatory submissions.

Information presented in Section VIII.C demonstrated that MON 95379 maize is no more susceptible to diseases or pests than commercially cultivated maize. Additionally, data presented in this petition support that, with the exception of the introduced trait, MON 95379 maize is phenotypically similar to conventional maize and is not expected to pose a greater plant pest risk than conventional maize. This section will focus on the potential impacts to agricultural and cultivation practices considering the intended use of small-scale breeding and seed increase. It is not anticipated that breeding and seed increase activities in the U.S. would have a notable impact on current practices such as the management of weeds, diseases, and insects except for the management of lepidopteran pests.

IX.B. Maize Breeding and Seed Production

An overview of U.S. maize production in the context of commercial production was provided in Bayer's petition for nonregulated status of MON 87429 maize (USDA-APHIS Petition #19-316-01p p.125). However, MON 95379 will not be commercialized in the U.S. but is intended to be cultivated in small-scale breeding, testing, and seed increase nurseries to develop seed for future products in South America as well as potential small-scale confined field trials for stack products in other locations, as described earlier.

Maize nurseries typically used for breeding and seed increase activities utilize practices to minimize pollen flow in order to produce high quality seeds. Those practices may include, but are not limited to: 660 feet spatial isolation or 21 day temporal isolation from other maize, planting border around nurseries (10 - 15 ft), maintaining fallow area (up to 50 ft) and hand-bagging (isolating) male flower (tassels) of plants targeted to be self-pollinated or used as a pollen donor. In hybrid seed production fields (commonly called isolated crossing blocks) female parental inbred rows (MON 95379 is expected to be deployed on female side of the pedigree) would be de-tasseled or utilize male sterility technologies.

Pre-season, in-season and post-harvest agronomic practices, including insect, disease or weed control management, crop rotation or volunteer management, will not differ for MON 95379 seed production when compared to the current maize practices implemented by nursery sites. Seed produced will not be used for commercial grain production in the United States.

IX.B.1. Management of Insect Pests

Bayer summarized major issues associated with the management of insect pests in its petition for nonregulated status for corn rootworm-protected maize MON 87411 maize (USDA-APHIS Petition #13-290-01p p166). In brief, insect pests are a significant source of damage to maize and are commonly addressed by biotechnology-derived insect-protection traits, insecticide treatment of seeds, soil or foliar application of insecticides, or use of crop rotation or other integrated pest management practices. The EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), regulates the distribution, sale, use and testing of pesticidal substances (including those produced in plants), that are intended to control insect pests.

Although MON 95379 contains the coding sequences for the Cry1B.868 and Cry1Da_7 proteins that provide protection against certain lepidopteran insect pests present in the U.S., Bayer is only seeking to cultivate MON 95379 in small-scale breeding, testing, and seed increase nurseries to develop seed for future products in South America as well as potential small-scale confined field trials for stack products in other locations, as described earlier.

IX.B.2. Management of Diseases and Other Pests

Bayer summarized major issues associated with the management of diseases and non-insect pests in its petition for nonregulated status for corn rootworm-protected maize MON 87411 maize (USDA-APHIS Petition #13-290-01p p171). MON 95379 does not contain disease protection traits, therefore the information on this subject is incorporated here by reference (USDA-APHIS Petition #13-290-01p p. 171).

MON 95379 was developed to improve the management of targeted lepidopteran insect pests and has no unique attributes for control of diseases or other pests. Environmental interactions observations in field studies indicated that the presence of the lepidopteran-protected trait did not meaningfully alter the susceptibility of MON 95379 maize to diseases or other pests (Section VIII.C). Therefore, no changes in current management practices for diseases or other pests in maize are anticipated for the intended use of MON 95379 maize relative to conventional maize.

IX.B.3. Weed Management

MON 95379 does not contain herbicide tolerance trait and is therefore no different from other non-herbicide tolerant maize in terms of its weed management considerations. However, the information on this subject is incorporated here by reference from its petition for nonregulated status for MON 87429 maize (USDA-APHIS Petition #19-316-01p p.130). In brief, annual and perennial weeds are considered to be the greatest pest problem in maize production (Aref and Pike, 1998). Weed control in maize is essential for optimizing yield because weeds compete with maize for light, nutrients, and moisture and can lead to reductions in yield (Knake et al., 1990).

IX.B.4. Crop Rotation and Volunteer Management Practices in Maize

MON 95379 activities in the United States will be limited to breeding and seed increase activities and will not be commercialized in the U.S. These breeding and seed production activities will take place at research and breeding facilities following typical recommended practices employed to achieve high quality seed and control volunteer maize as needed to maintain and assure product quality. The intended use of MON 95379 maize in the U.S. is not expected to impact crop rotation practices or volunteer management practices in maize.

IX.B.5. Stewardship of MON 95379 Maize

Bayer develops effective products and technologies that deliver value to growers and conserve resources that agriculture depends on and is committed to assuring that its products and technologies are safe and environmentally responsible. Bayer demonstrates this commitment by implementing product stewardship processes throughout the lifecycle of a product and by participation in the Excellence Through Stewardship® (ETS) Program[13] (BIO, 2010). These policies and practices include rigorous field compliance and quality management systems and verification through auditing.

As an integral component of fulfilling this global stewardship commitment, Bayer will seek biotechnology regulatory approvals for MON 95379 maize in all important maize import countries with a functioning regulatory system to assure global compliance and support the flow of international trade. These actions will be consistent with the ETS Guide for Product Launch Stewardship of Biotechnology-Derived Plant Products (ETS, 2018) and Biotechnology Innovation Organization (BIO) Policy on Product Launches (BIO, 2012).

Bayer is also committed to following industry best practices on seed quality assurance and control to ensure the purity and integrity of MON 95379 maize hybrid seed. As with all of Bayer's products, before commercializing MON 95379 maize in any country, a MON 95379 maize detection method will be available via a third-party vendor to maize producers, processors, and buyers regardless of whether they have purchased MON 95379 or not.

IX.C. Summary and Conclusion: Impact of the Introduction of MON 95379 Maize on Agricultural Practices

MON 95379 was developed to improve the management of targeted lepidopteran insect pests for growers in South America and has no unique attributes for control of diseases or other pests. MON 95379 will not be commercialized in the U.S. and is therefore not expected to have adverse impacts on current agronomic, cultivation and management practices for commercial grain or hybrid maize seed production. Activities will be limited to small-scale breeding, testing and seed increases in the U.S., with typical production practices to produce high quality seed and/or data. Evaluation of the agronomic and phenotypic characteristics of MON 95379 support that, with the exception of the introduced trait, MON 95379 maize is phenotypically similar to conventional maize and is not expected to have an effect on practices used in small scale breeding and seed activities.

[13] www.excellencethroughstewardship.org/

X. PLANT PEST ASSESSMENT

X.A. Introduction

This section provides a brief review and assessment of the plant pest potential of MON 95379 and its potential impact on agronomic practices and the environment. USDA-APHIS has responsibility, under the Plant Protection Act (PPA) (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. Prior regulations codified in 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article is unlikely to pose a greater plant pest risk than conventional maize, the petition is granted, thereby allowing introduction without the need for permit or notification.

According to the PPA, the definition of "plant pest" includes the living stage of any of the following, or a similar article, that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; or (G) an infectious agent or other pathogens (7 U.S.C. § 7702[14]).

The regulatory endpoint under the USDA-APHIS regulations for biotechnology-derived crop products is not zero risk, but rather a determination that deregulation of the article in question is not expected to pose a greater plant pest risk than conventional maize. Information in this petition related to plant pest risk characteristics includes: 1) mode-of-action and changes to plant metabolism; 2) composition; 3) expression and characteristics of the gene product; 4) potential for weediness of the regulated article; 5) potential impacts to NTOs beneficial to agriculture and no effect on threatened and endangered species; 6) abiotic stressor, disease and pest susceptibilities; 7) impacts on agronomic practices; and 8) impacts on the weediness of any other plant with which it can interbreed, as well as the potential for gene flow. Using the assessment above, the data and analysis presented in this petition led to a conclusion that MON 95379 is unlikely to pose a greater plant pest risk than conventional maize, and therefore should no longer be subject to regulation under 7 CFR § 340.

X.B. Plant Pest Assessment of MON 95379 and Expressed Cry1B.868 and Cry1Da_7 Proteins

This section summarizes the details of the genetic insert, characteristics of the genetic modification, and safety and expression of the Cry1B.868 and Cry1Da_7 protein expressed in MON 95379 used to evaluate the food, feed, and environmental safety of MON 95379.

X.B.1. Characteristics of the Genetic Insert and Expressed Protein

X.B.1.1. Genetic Insert

As described in Section III.B, MON 95379 was developed by *Agrobacterium*-mediated transformation of maize embryos using plasmid vector PV-ZMIR522223. Characterization of the DNA insert in MON 95379 was conducted using a combination of sequencing, PCR, and bioinformatics methods. The results of this characterization demonstrate that MON 95379 contains one copy of the intended transfer DNA containing the *cry1B.868* and *cry1Da_7* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple breeding generations. These methods also confirmed that no vector backbone or other unintended plasmid sequences (from PV-ZMIR522223 or PV-ZMOO513642 (the "Cre line" transformation vector)) are present in MON 95379. Furthermore it was confirmed that the *cp4 epsps* selectable marker cassette, excised by Cre recombinase, is not present in MON 95379. Additionally, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 95379 to the sequence of the insertion site in conventional maize. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 95379 upon DNA integration.

X.B.1.2. Mode-of-Action

The mode of action of Cry proteins has been extensively studied and is well-documented (Gill et al., 1992; OECD, 2007; Schnepf et al., 1998; Vachon et al., 2012). The mode of action for Cry1B.868 and Cry1Da_7 have been assessed (Wang et al., 2019) and follows the same general steps as other *Bt* insecticidal proteins currently in commercial use for insect crop protection (Jerga et al., 2016). The uptake, activation, receptor binding, and pore formation steps that lead to insect toxicity are conserved in Cry1B.868 and Cry1Da_7. The receptor binding of Cry1B.868 and Cry1Da_7 confers specificity to the targeted lepidopteran insect pests (Wang et al., 2019).

X.B.2. Protein Safety and Expression Levels

The safety and expression of the Cry1B.868 and Cry1Da_7 proteins are detailed in Section V. MON 95379 Cry1B.868 and Cry1Da_7 protein levels in leaf, root, silk, pollen, forage, forage-root and grain of MON 95379 were determined using an enzyme-linked immunosorbent assay (ELISA) as described in Appendix E. The mean Cry1B.868 protein level in MON 95379 across all sites was the highest in leaf tissue (OSL 1) at 630 µg/g dw and lowest in forage root at 22 µg/g dw. The mean Cry1Da_7 protein levels in MON 95379 across all sites was highest in leaf (OSL1) at 92 µg/g dw and lowest in grain at <LOQ µg/g dw.

The Cry1B.868 and Cry1Da_7 proteins expressed by MON 95379 maize are PIPs and therefore are regulated by the U.S. Environmental Protection Agency (EPA). The data for the safety assessment of Cry1B.868 and Cry1Da_7 proteins in MON 95379 were provided to EPA in a petition to establish a permanent exemption from the requirement of a tolerance for Cry1B.868 and Cry1Da_7 proteins in or on food and feed commodities of maize. The physicochemical and functional characteristics of the MON 95379-produced Cry1B.868 and Cry1Da_7 proteins were determined and equivalence between MON 95379-produced Cry1B.868 and Cry1Da_7 and *Bt*-produced Cry1B.868 and Cry1Da_7 proteins was demonstrated. Therefore, *Bt*-produced

Cry1B.868 and Cry1Da_7 are appropriate test substances for the human, mammalian and NTO safety studies. Expression studies demonstrated that Cry1B.868 and Cry1Da_7 proteins are expressed at low levels in grain. Assessments of the allergenic and toxic potential of the Cry1B.868 and Cry1Da_7 proteins found that the Cry1B.868 and Cry1Da_7 proteins do not pose a significant allergenic risk and were not similar to known toxins. In addition, the donor organism for the MON 95379 Cry1B.868 and Cry1Da_7 coding sequence is from *Bacillus thuringiensis* which is ubiquitous in the environment and is not known for allergenicity and human or animal pathogenicity. The Cry1B.868 and Cry1Da_7 proteins are rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) and demonstrate no acute oral toxicity in mice at the highest level tested, which far exceeds any exposure scenario. Based on the above information, the consumption of the Cry1B.868 and Cry1Da_7 proteins from MON 95379 or its progeny is considered safe for humans and mammals. Taken together, the results of these analyses support a determination that MON 95379 is no more likely to pose a plant pest risk than conventional maize.

X.B.3. Compositional Characteristics

Compositional comparisons of MON 95379 to conventional control followed considerations relevant to the compositional quality of maize as defined by the OECD consensus document (OECD, 2002a) were presented in Section VI. Grain samples were analyzed for levels of key nutrients including proximates (protein, fat, ash, moisture), essential amino acids (10 components), linoleic acid, carbohydrates by calculation, acid detergent fiber (ADF) and neutral detergent fiber (NDF). The anti-nutrients analyzed in grain were phytic acid and raffinose. Forage samples were analyzed for levels of proximates, carbohydrates by calculation, and fiber (ADF, NDF). In all, 27 different components were analyzed. Moisture values for grain and forage were measured for conversion of components from fresh to dry weight but were not statistically analyzed. Therefore, 25 different components were statistically analyzed.

Of the 25 components statistically assessed, 17 showed no significant differences between MON 95379 and the conventional control. Eight components in grain (protein, carbohydrate, isoleucine, leucine, methionine, phenylalanine, threonine and valine) showed a significant difference between MON 95379 and the conventional control. For these eight components, the mean difference in the component values between MON 95379 and the conventional control was less than the range value of the conventional control. The MON 95379 mean component value was also within the range of values observed in the literature and the ILSI-CCDB. These data indicated that the statistically significant differences in grain were not compositionally meaningful from a food and feed safety perspective. There were no statistically significant differences ($p < 0.05$) between MON 95379 and the conventional control forage components observed.

These results support the overall conclusion that MON 95379 was not a major contributor to variation in key nutrient and anti-nutrient levels in maize grain and forage and confirmed the compositional equivalence of MON 95379 to the conventional control in levels of these components.

X.B.4. Assessment of Potential Impacts on Non-Target Organisms Beneficial to Agriculture and Threatened and Endangered Species

Prior to conducting a risk assessment for MON 95379 maize to beneficial NTOs, the proposed areas for cultivation of MON 95379 maize, the MOA and the spectrum of insecticidal activity of Cry1B.868 and Cry1Da_7 proteins, as well as expression analyses of the Cry1B.868 and Cry1Da_7 proteins produced by MON 95379 tissues were evaluated (Section VII.A). The results of the activity spectrum studies indicate that, similar to previously commercialized Cry1 proteins, activity of Cry1B.868 and Cry1Da_7 proteins is limited to lepidopteran insects and effects are not expected on NTOs beneficial to agriculture. An evaluation of potential off-crop exposure to MON 95379 pollen indicated significant exposure to non-target lepidopteran species is not anticipated and therefore poses minimal risk to non-target Lepidoptera. Results from Tier I NTO testing demonstrated no effects on survival from exposure to the Cry1B.868 and Cry1Da_7 proteins at levels well above those found in MON 95379 maize and in the environment (Section VII.C). Calculated margins of exposure based on LC_{50} values and conservative environmental concentrations were >11 for all species tested with the exception of *C. maculata* where the MOE for Cry1Da_7 was >7, indicating low risk to NTOs at field expression levels. A threatened and endangered species assessment focused on listed Lepidoptera in the states of Nebraska, Hawaii, and Iowa, where MON 95379 will primarily be cultivated, resulted in the conclusion of no effect for currently listed lepidopteran species.

X.B.5. Phenotypic, Agronomic, and Environmental Interaction Characteristics

An extensive set of comparative plant characterization data were used to assess whether the introduction of lepidopteran-protected trait altered the plant pest potential of MON 95379 compared to the conventional control (Section VIII). Phenotypic, agronomic, and environmental interactions characteristics of MON 95379 were evaluated and compared to those of the conventional control. As described previously, these assessments included seed germination and dormancy characteristics in the laboratory as well as phenotypic and agronomic characteristics and plant responses to abiotic stressors, diseases, and arthropod pests in the field. Results from all phenotypic, agronomic, and environmental interaction assessments demonstrated that MON 95379 does not possess increased weedy characteristics or increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests compared to the conventional control. Taken together, the assessments support a determination that MON 95379 is no more likely to pose a plant pest risk than conventional maize.

X.B.5.1. Seed Dormancy and Germination

A comparative assessment of seed germination and dormancy characteristics was conducted on MON 95379 and the conventional control. The results of this assessment, including the lack of significant differences and particularly the lack of increased hard seed, support the conclusion that the introduction of the lepidopteran-protected trait does not result in increased weediness or plant pest risk of MON 95379 compared to conventional maize.

X.B.5.2. Phenotypic and Agronomic Assessment

Evaluations of phenotypic and agronomic characteristics in the field are useful for assessing characteristics typically associated with weediness, such as lodging and seed loss. The characteristics early stand count, days to flowering, plant height, days to maturity, lodging, seed loss, final stand count, moisture, seed weight, and yield were assessed. The lack of observed differences between MON 95379 and the conventional control for phenotypic and agronomic characteristics across sites supports the overall conclusion that the introduction of the lepidoptern protected trait does not result in increased weediness or plant pest risk of MON 95379 compared to conventional maize.

X.B.5.3. Response to Abiotic Stressors, Diseases, and Arthropod Pests

In an assessment of plant response to abiotic stressors, diseases, and arthropod pests, no differences were observed between MON 95379 and the conventional control for any of the 96 observations for each stressor category across eight sites (Section VII.C.2). The lack of observed differences between MON 95379 and the conventional control for plant responses to abiotic stressors, diseases, and arthropod pests in multiple field environments supports the overall conclusion that the lepidopteran-protected trait does not result in increased plant pest risk of MON 95379 compared to conventional maize.

X.C. Weediness Potential of MON 95379

Although grown extensively throughout the world, maize is not considered a threat to invade natural or agricultural ecosystems. Maize does not establish self-sustaining populations outside of cultivation (Crawley et al., 2001; OECD, 2003; Raybould et al., 2012). This lack of weediness may reflect its poor competitive ability (Olson and Sander, 1988), lack of seed dormancy, and barriers to seed dispersal, as maize cobs retain seed and are covered in a husk (Wilkes, 1972). A number of other characteristics common in weeds, such as rapid flowering following emergence, are lacking in maize (Keeler, 1989). Traits often associated with weediness are typically not selected for during domestication and subsequent breeding and selection. Similarly, the history of maize breeding and production does not indicate there are any changes in the characteristics of maize that would increase the weediness of the crop. Even if kernels of maize are distributed within a field or along transportation routes from the fields to storage or processing facilities, self-sustaining volunteer maize populations are not found growing in fields, fence rows, ditches, or roadsides.

In comparative studies between MON 95379 and a conventional control, germination and dormancy, phenotypic and agronomic, environmental interaction were evaluated (Section VIII)

for changes that could impact the plant pest potential, and in particular, plant weediness potential. Results of these evaluations show that there were no compositional differences that were biologically meaningful between MON 95379 and the conventional control for characteristics (percentage viable hard seed, lodging, and seed loss) typically associated with weediness. Furthermore, field observations of MON 95379 and the conventional control indicated no differences in plant responses to abiotic stressors, diseases, or arthropod pests. Collectively, these findings support the conclusion that MON 95379 is no more likely to become a weed than conventional maize.

X.D. Potential for Pollen Mediated Gene Flow and Introgression

Pollen mediated gene flow (often referred to as cross pollination) occurs when pollen of one plant fertilizes ovules of a second plant. Pollen mediated gene flow is affected by both biotic and abiotic factors such as plant biology, pollen biology/volume, plant phenology, overlap of flowering times, proximity of the pollen source and sink, ambient conditions such as temperature and humidity, and field architecture. Pollen mediated gene flow is a natural biological process, and therefore does not constitute an environmental risk in and of itself.

Introgression is a process whereby one or more genes successfully incorporate into the genome of a recipient plant. Pollen mediated gene flow and gene introgression must be considered in the context of the transgenes inserted into the biotechnology-derived plant, and the likelihood that the presence of the transgenes and their subsequent transfer to recipient plants and plant populations will result in increased plant pest potential.

Under the proposed terms of an EPA seed increase registration application, MON 95379 maize will be cultivated for small-scale breeding, testing, and seed increase-related activities as well as potential small-scale confined field trials for stack products in other locations, as described earlier. . The potential for gene flow and introgression from deregulation of MON 95379 is discussed in greater detail below.

X.D.1. Hybridization with Cultivated Maize

Maize is a wind pollinated species with plant morphology that facilitates cross pollination. Therefore, relatively high levels of pollen-mediated gene flow can occur in this species at short distances (Jones and Brooks, 1950). Some biotic and abiotic factors that may influence the amount of pollen-mediated gene flow in maize include: (1) wind direction and speed; (2) distance between the pollen-source and pollen-recipient plants; (3) environmental factors that may impact pollen viability and dispersal (e.g. temperature and relative humidity); (4) duration of pollen shed and (5) floral synchrony between pollen donor and pollen recipient.

The results from several studies conducted on the extent of pollen-mediated gene flow between maize fields demonstrate consistent trends regardless of the experimental design, world region, or detection method. The amount of pollen-mediated gene flow is greatest within the first few meters and decreases sharply with increasing distance from the pollen source (Table X-1). The distance >200 m (660 feet) is used for managing gene flow during breeding, seed production, identity preservation or other applications; in addition, it forms the basis for the USDA-APHIS performance standards for maize. All U.S. testing and production of regulated MON 95379 seed

or grain have been conducted under USDA notification or release permits according to these standards. Since no meaningful differences were observed for MON 95379 compared to conventional maize in composition or nutritional value, or in pest/weed potential in field evaluations, no adverse effects are expected from gene flow from small-scale MON 95379 breeding or seed production nurseries to other maize.

Table X-1. Summary of Published Literature on Maize Cross Pollination

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
1 5 10 14 19 24 28 33 36	19.0 2.6 2.0 0.6 0.4 0.3 0.5 0.3 0.1	Three-year study with two to three sites per year. Outcrossing was detected by yellow seeds in the white-seeded pollen recipient Values are averaged over site-years for the downwind direction.	Canada	(Ma et al., 2004)
24–32 60–62 123–125 244–254 486–500 743–745	0.69 0.23 0.08 0.02 0.005 0.002	Two-year study with two sites per year. Outcrossing was detected by purple seeds in the yellow-seeded pollen recipient. Values are the greatest observed between years for the site where outcrossing was most prevalent.	USA	(Halsey et al., 2005)
1 10 35 100 150 200 250	29.9 2.5 0.4 0.05 0.03 0.03 0.03	Two-year study with one site per year. Outcrossing was detected by yellow seeds in the non-transgenic white-seeded pollen recipient and confirmed by tests for a glyphosate tolerance gene in the yellow seeds. Values are the greatest between years as averaged across all directions from the pollen source.	USA	(Goggi et al., 2006)

Table X-1. Summary of Published Literature on Maize Cross Pollination (continued)

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
0 2 5 10 20 40 80	12.65 8.81 2.33 3.69 1.05 0.74 0.65	One-year study with one site. Outcrossing was detected by yellow seeds in the white-seeded pollen recipient. Values are the greatest reported at listed distances from the edge of two sampling areas, one adjacent to and one 10 m from the pollen source. The values are half of those observed to reflect flow of a hemizygous gene.	Spain	(Pla et al., 2006)
0 2 5 10 20 25 50 100 150	0.74 0.66 0.35 0.27 0.16 0.14 0.12 0.10 0.12	Three-year study with a total of 55 sites. Outcrossing was quantified on ground samples of nontransgenic pollen recipient grain using a PCR assay for the *pat* herbicide tolerance gene. Values are means for percentage GM DNA for distances with >30 samples.	UK	(Weekes et al., 2007)

Table X-1. Summary of Published Literature on Maize Cross Pollination (continued)

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
1 5 10 15 30 60 100 150 200 300	44 16 10 5 1 1 0 0 0 0	Two-year study with one site per year. Outcrossing was detected by herbicide tolerance and PCR tests for an herbicide tolerance transgene in progeny of the non-transgenic pollen recipient. Values are the greatest between years as averaged across all directions from the pollen source.	China	(Zhang et al., 2011)
1 2 4 8 12 16 20 25	12.9 4.6 2.7 1.4 1.0 0.8 0.5 0.5	Three-year study with a total of eight sites. Outcrossing was detected by yellow seeds in the white-seeded pollen recipient. Values are averaged over all directions and sites.	Mexico	(Baltazar et al., 2015)

X.D.2. Hybridization with Teosinte

For gene flow to occur by typical sexual transmission, the following conditions must exist: (1) the two parents must be sexually compatible; (2) there must be flowering synchrony between the pollen source and pollen recipient; (3) the plants must be within sufficient proximity to each other; and (4) suitable environmental factors, such as relative humidity, temperature, or wind, must be present.

Maize is sexually compatible with certain species or subspecies of teosinte. Although teosinte is not native to the U.S., a compatible subspecies of annual teosinte (*Zea mays* subsp. *mexicana*) is reported to have feral populations in Florida, Alabama, and Maryland (USDA-NRCS, 2019).

Hybrids of maize and teosinte are not expected in the United States. In a study of maize and *Zea mays* subsp. *mexicana*, very few ovules (approximately 1-2%) produced seed after hand pollination with pollen from the other subspecies (Baltazar et al., 2005). Natural hybridization rates for maize and *Zea mays* subsp. *mexicana* have also been reported to be low (≤0.2% of progeny) (Ellstrand et al., 2007). A genetic barrier to hybridization of maize and teosinte has been described (Evans and Kermicle, 2001) and if present may limit hybridization rates of maize and *Zea mays* subsp. *mexicana*. In addition, differences in developmental and morphological factors

(Baltazar et al., 2005), potential differences in flowering time, and limited geographical distribution of teosinte make natural crosses and gene introgression from maize into teosinte unlikely in the U.S.

X.D.3. Hybridization with *Tripsacum*

Tripsacum is a genus with 15 recognized species (Zuloaga et al., 2003). There are three species of *Tripsacum* that are native to the U.S.: *T. floridanum* (Florida gamagrass), *T. lanceolatum* (Mexican gamagrass), and *T. dactyloides* (Eastern gamagrass) (USDA-NRCS, 2019). Two additional species have introduced populations: *T. latifolium* (wideleaf gamagrass) and *T. fasciculatum* (Guatemalan gamagrass) (USDA-NRCS, 2019).

Tripsacum floridanum (Florida gamagrass) naturally grows in the extreme southern Florida counties of Miami-Dade, Collier, Martin, and Monroe (USDA-NRCS, 2019; Wunderlin et al., 2019). Florida gamagrass has been described as rare and occurring in "low, rocky pinelands" (Blakey et al., 2007) and is categorized as a threatened species in Florida (USDA-NRCS, 2019). *Tripsacum lanceolatum* (Mexican gamagrass) has been reported in Arizona and New Mexico (USDA-NRCS, 2019) and is found on "stream banks or moist cliffs" (de Wet and Harlan, 1978). *Tripsacum dactyloides* (eastern gamagrass) is found primarily throughout the eastern U.S. It is categorized as endangered in Massachusetts and Pennsylvania, threatened in New York, and special concern in Rhode Island (USDA-NRCS, 2019). *Tripsacum fasciculatum* (Guatemalan gamagrass) and *T. latifolium* (wideleaf gamagrass) and are present as introduced populations in Puerto Rico (USDA-NRCS, 2019).

To our knowledge, hybrids between maize and *Tripsacum* do not occur in nature. The formation of hybrids between maize and *Tripsacum* species requires human intervention under specific controlled laboratory conditions, and the hybrids exhibit male sterility that is not resolved by several backcrosses to maize (Russell and Hallauer, 1980). Empirical data showed that "no evidence of gene flow from transgenic maize to eastern gamagrass in nature was observed even though the two species have grown in close proximity for years and have had ample opportunities for outcrossing" (Lee et al., 2017). Thus, no species of *Tripsacum* is expected to form viable hybrid progeny with maize under natural conditions.

In summary, although hybrids between maize and *Tripsacum* have been produced using specialized laboratory techniques, there is no evidence of hybrid existence in nature. Therefore, gene flow from maize to any *Tripsacum* species is extremely unlikely. Under natural conditions, as is the case with conventional maize, pollen-mediated gene flow from MON 95379 to any species of *Tripsacum* is not expected.

X.E. Transfer of Genetic Information to Species with which Maize Cannot Interbreed (Horizontal Gene Flow)

Bayer is unaware of any reports regarding the unaided transfer of genetic material from maize species to other sexually-incompatible plant species. The likelihood for horizontal gene flow to occur is exceedingly small, a conclusion also reached by USDA (APHIS-BRS Final PPRA, p. 31 for Petition #15-113-01p). Therefore, potential ecological risk associated with horizontal gene flow from MON 95379 due to the presence of lepidopteran-protected trait are not expected. The

consequence of horizontal gene flow of the MON 95379 trait into other plants that are sexually-incompatible is negligible since, as data presented in this petition confirm, the genes and traits confer no increased plant pest potential to maize. Thus, in the highly unlikely event that horizontal gene transfer was to occur, the presence of the MON 95379 trait would not be expected to increase pest potential in the recipient species.

X.F. Potential Impact on Maize Agronomic Practices

An assessment of current maize agronomic practices was conducted to determine whether the cultivation of MON 95379 has the potential to impact current maize management practices (Section IX). MON 95379 will not be commercialized in the U.S. and therefore will not impact U.S. commercial maize agricultural practices. It is intended to be cultivated in small scale breeding, testing, and seed increase nurseries to develop seed for future products in South America. In the future, MON 95379 stack products may also be planted in confined, small-scale field trials that would support subsequent global regulatory submissions.Maize fields utilized for small-scale breeding, testing and seed increase activities are typically highly managed agricultural areas that are dedicated to these purposes. Other than the specific insertion of the Cry1B.868 and Cry1Da_7 coding sequence that provide protection against targeted lepidopteran insect pests MON 95379 is similar to other conventional maize hybrids.

The data presented demonstrate that MON 95379 is similar to conventional maize in its phenotypic and agronomic characteristics, and has levels of susceptibility to abiotic stressors, diseases, and arthropod pests comparable to other conventional maize. Based on this assessment, the intended use of MON 95379 for breeding and seed increase activities, MON 95379 will not have an impact on current U.S. maize agronomic or cultivation practices, and is not likely to have adverse impacts on practices used in small-scale breeding/seed increase activities, or lead to an increased plant pest potential compared to conventional maize.

X.G. Conventional Breeding with Other Biotechnology-derived or Conventional Maize

Several biotechnology-derived maize products have been deregulated or are under consideration for deregulation. Once deregulated, MON 95379 may be bred with these deregulated biotechnology-derived maize products, as well as with conventional maize, creating new improved hybrids. APHIS has determined that none of the individual biotechnology-derived maize products it has previously deregulated displays increased plant pest characteristics. APHIS has also concluded that any progeny derived from crosses of these deregulated biotechnology-derived maize products with conventional or previously deregulated biotechnology-derived maize are unlikely to exhibit new plant pest properties. This presumption, that combined-trait biotechnology products are unlikely to exhibit new characteristics that would pose new plant pest risks or potential environmental impacts not observed in the single event biotech product, is based upon several facts, namely: 1) stability of the genetic inserts is confirmed in each approved biotech-derived maize product across multiple generations (See Section IV.E for MON 95379 data); 2) stability of each of the introduced traits is continually and repeatedly assessed by plant breeders as new combined-trait hybrids are created and tested over multiple seasons prior to commercialization; 3) combined-trait products are developed using the well-established process of conventional breeding that has been safely used for thousands of years to generate new varieties (Cellini et al., 2004; NRC, 2004; WHO, 1995); 4) worldwide organizations, such as World Health

Organization, Food and Agriculture Organization/World Health Organization, International Seed Federation, CropLife International and U.S. FDA, conclude that the safety of the combined-trait product can be based on the safety of the parental GE events (CLI, 2005; FAO-WHO, 1996; ISF, 2005; U.S. FDA, 2001; WHO, 1995); and 5) practical applications in the field have shown that two unrelated biotechnology traits combined together by conventional breeding do not display new characteristics or properties distinct from those present in the single event biotech products (Brookes and Barfoot, 2012; Clawson et al., 2019; James, 2010; Jose et al., 2020; Lemaux, 2008; Pilacinski et al., 2011; Sankula, 2006).

Therefore, based on the considerations above and the conclusion that MON 95379 is no more likely to pose a plant pest risk than conventional maize, it can be concluded that any progeny derived from crosses between MON 95379 and conventional maize or other maize with deregulated biotechnology-derived events are no more likely to pose a plant risk than conventional maize.

X.H. Summary of Plant Pest Assessments

A plant pest, as defined in the Plant Protection Act, is the living stage of any of the following that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; (G) an infectious agent or other pathogens; or (H) any article similar to or allied with any of the articles specified in the preceding subparagraphs (7 U.S.C. § 7702[14]). Characterization data presented in Sections III through IX of this petition confirm that MON 95379, with the exception of the lepidopteran-protected trait, is not meaningfully different from conventional maize in terms of plant pest potential. Bayer is not aware of any other study results or observations associated with MON 95379 that would suggest an increased plant pest risk would result from its limited cultivation for small-scale breeding, testing, and seed increase-related activities.

The plant pest assessment was based on multiple lines of evidence developed from a detailed characterization of MON 95379 compared to conventional maize, followed by a risk assessment on detected differences. The plant pest risk assessment in this petition was based on the following lines of evidence: 1) insertion of a single functional copy of the Cry1B.868 and Cry1Da_7 cassette; 2) characterization and safety of the expressed products; 3) compositional equivalence of MON 95379 grain and forage compared to a conventional control; 4) phenotypic, agronomic, and environmental interactions characteristics demonstrating no increased plant pest potential compared to conventional maize; 5) minimal risk to NTOs including organisms beneficial to agriculture and no effect on threatened and endangered species 6) familiarity with maize as a cultivated crop and 7) no impact on U.S. commercial maize agricultural practices as MON 95379 will not be commercialized in the U.S. and no greater likelihood to impact practices used in small-scale breeding, testing and seed increase activities, agronomic practices, cultivation practices, or the management of weeds, diseases and pests, than conventional maize.

Based on the data and information presented in this petition, it is concluded that MON 95379 is unlikely to pose a greater plant pest risk than conventional maize. Therefore, Bayer Company requests a determination from USDA-APHIS that MON 95379 and any progeny derived from

crosses between MON 95379 and other de-regulated maize events be granted nonregulated status under 7 CFR part 340.

XI. ADVERSE CONSEQUENCES OF INTRODUCTION

Bayer knows of no study results or observations associated with MON 95379 indicating that there would be adverse consequences from the intended use of MON 95379 for breeding and seed increase activities. MON 95379 produces the Cry1B.868 and Cry1Da_7 proteins. The safety of the MON 95379 Cry1B.868 and Cry1Da_7 proteins, which have been fully characterized, has been thoroughly assessed in this submission. The data for the safety assessment of Cry1B.868 and Cry1Da_7 and MON 95379 is being provided to EPA to support the establishment of a tolerance exemption for residues of Cry1B.868 and Cry1Da_7 in food and feed. As demonstrated by field results and laboratory tests, the only phenotypic differences between MON 95379 and conventional maize is presence of lepidopteran-protected traits which protect against feeding damage caused by targeted lepidopteran insect pests.

The data and information presented in this petition demonstrate that MON 95379 is unlikely to pose a greater plant pest risk compared to conventional maize. This conclusion is reached based on multiple lines of evidence developed from a detailed characterization of the product compared to conventional maize, followed by risk assessment on detected differences. The characterization evaluations included molecular analyses, which confirmed the insertion of one copy of the intended DNA containing the *cry1B.868* and *cry1Da_7* expression cassette that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations.

Analysis of key nutrients and anti-nutrients of MON 95379 demonstrate that MON 95379 is compositionally equivalent to conventional maize. The phenotypic evaluations, including an assessment of seed germination and dormancy characteristics, phenotypic and agronomic characteristics, environmental interactions also indicated MON 95379 is unchanged compared to conventional maize. There is no indication that MON 95379 would have an adverse impact on non-target organisms (NTOs), including those beneficial to agriculture, and would have no effect on threatened or endangered species under the intended use of the product. Therefore, based on the lack of increased pest potential compared to conventional maize, the risks for humans, animals, and other NTOs from MON 95379 are negligible.

MON 95379 will not be commercialized in the United States and will therefore have no impact on U.S. commercial maize agricultural practices. The intended use of MON 95379 for small-scale breeding, testing and seed increase activities will not adversely impact cultivation practices or the management of weeds, diseases, and pests in maize seed production systems. Activities will be limited to breeding and seed increase in Nebraska, Hawaii and Iowa on a maximum total of 100 acres per growing season and small-scale, confined field trials (in other locations, as described earlier) to collect needed regulatory data to support global regulatory submissions of future products stacked with MON 95379. Seed production activities will take place at research and breeding facilities following typical recommended practices employed to achieve high quality seed. Based on the data and information presented in this petition, it is concluded that, like previously deregulated biotechnology-derived maize, MON 95379 is unlikely to pose a greater plant pest risk than conventional maize.

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Appendix A: USDA Notifications and Permits

Field trials of MON 95379 have been conducted in the U.S. since 2015. The protocols for these trials include field performance, breeding and observation, agronomics, and generation of field materials and data necessary for this petition. In addition to the MON 95379 phenotypic assessment data, observational data on pest and disease stressors were collected from these product development trials. The majority of the final reports have been submitted to the USDA. However, some final reports, mainly from the 2019 season, are still in preparation. A list of trials conducted under USDA notifications or permits and the status of the final reports for these trials are provided in Table A-1.

Table A-1. USDA Notifications and Permits Approved for MON 95379 and Status of Trials Planted under These Notifications

Field Trial Year	USDA No.	Effective Date	Trial Status	Release State	Sites
2015	15-028-101rm	6/1/2015	Submitted to USDA	HI	1
				PR	1
	15-120-112rm	9/1/2015	Submitted to USDA	HI	2
2016	15-120-112rm	9/1/2015	Submitted to USDA	HI	1
				PR	1
	15-289-113rm	3/2/2016	Submitted to USDA	HI	2
				PR	1
	15-306-101rm	3/15/2016	Submitted to USDA	IA	4
				IL	5
				IN	1
				KS	3
	15-306-107rm	3/15/2016	Submitted to USDA	AR	1
				IL	6
				NE	4
				TN	2
	16-117-104rm	9/1/2016	Submitted to USDA	HI	1
	16-182-104rm	12/1/2016	Submitted to USDA	HI	1

Table A-1. USDA Notifications and Permits Approved for MON 95379 and Status of Trials Planted under These Notifications (continued)

2017	16-117-112rm	9/1/2016	Submitted to USDA	PR	1
	16-302-110rm	3/1/2017	Submitted to USDA	HI	1
	16-302-111rm	3/1/2017	Submitted to USDA	HI	1
	16-315-101rm	3/15/2017	Submitted to USDA	IA	10
				IL	18
	16-315-105rm	3/15/2017	Submitted to USDA	AR	2
				KS	5
				NE	6
				TN	2
	17-038-102n	3/7/2017	Submitted to USDA	AL	1
				NC	1
				TX	2
	17-115-108rm	9/1/2017	Submitted to USDA	HI	1
	17-115-111rm	9/1/2017	Submitted to USDA	HI	1

Table A-1. USDA Notifications and Permits Approved for MON 95379 and Status of Trials Planted under These Notifications (continued)

2018	17-304-105rm	3/1/2018	Submitted to USDA	HI	2
	17-304-107rm	3/1/2018	Submitted to USDA	HI	1
	17-304-108rm	3/1/2018	Submitted to USDA	HI	1
	17-320-101rm	3/15/2018	Submitted to USDA	IL	2
				NE	1
	17-320-102rm	3/15/2018	Submitted to USDA	IA	6
				IL	8
				IN	1
				NE	2
	18-065-105n	4/5/2018	Submitted to USDA	IA	2
				IL	1
				IN	1
				NC	1
				NE	1
				OH	1
				PA	1
				TX	1
	18-065-106n	4/5/2018	Submitted to USDA	IA	1
				IL	2
				NE	1
	18-122-103rm	9/1/2018	Submitted to USDA	HI	1
	18-122-106rm	9/1/2018	Submitted to USDA	HI	2
	18-144-104n	6/25/2018	Submitted to USDA	GA	1
	18-318-102rm	11/30/2018	Submitted to USDA	IA	1

Table A-1. USDA Notifications and Permits Approved for MON 95379 and Status of Trials Planted under These Notifications (continued)

	18-122-101rm	9/1/2018	Submitted to USDA	HI	1
	18-207-101rm	12/1/2018	Submitted to USDA	HI	1
	18-305-104rm	3/1/2019	In Progress	HI	1
	18-318-104rm	3/15/2019	In Progress	IL	1
2019	18-347-107rm	4/1/2019	In Progress	IA	3
				IL	3
				IN	1
				NC	2
				NE	1
				OH	1
				PA	1
	19-022-102rm	6/1/2019	In Progress	HI	1
	19-115-101rm	9/1/2019	In Progress	HI	2

Appendix B: Overview, Materials, Methods and Supplementary Results for Molecular Analyses of MON 95379

B.1. Test Substance

The test substance in this study was MON 95379. Five breeding generations of MON 95379 were used to assess the stability of the T-DNA insert. Genomic DNA for use in this study was extracted from seed tissue listed in the table below.

MON 95379 Breeding Generation	Seed ID
F4	11479057
F5	11479060
F4F1	11478830
F5F1	11479279
F6F1	11493009

B.2. Control Substances

The control substance is the conventional crop variety which is the same genetic background as the test substances. Genomic DNA was extracted from seed as listed in the table below.

Control Substance	Seed ID	Breeding Generations
LH244	11479052	F4, F5
LH244 × HCL617	11479278	F4F1, F5F1, F6F1

B.3. Reference Substance

The reference substances were plasmid vector PV-ZMIR522223 and PV-ZMOO513642, which were used to develop MON 95379 and a Cre recombinase expressing line. Whole plasmid DNA and its sequence served as a positive control for sequencing and bioinformatic analyses. The identity of the reference plasmid was confirmed by sequencing within the study. Documentation of the confirmation of the plasmid vector identity was archived with the raw data. Appropriate molecular weight markers from commercial sources were used for size estimations on agarose gels. The unique identity of the molecular weight markers was documented in the raw data.

B.4. Characterization of Test, Control, and Reference Substances

The Study Director reviewed the chain-of-custody forms to confirm the identity of the test and control substances prior to the use of these materials in the study. Further confirmation of test and control substances identity was determined by sequencing in the study. Test, control, and reference DNA as well as sequencing library substances were considered stable during storage if they yield interpretable signals in sequencing experiments, or did not appear visibly degraded on the stained gels (DNA) or sufficient cluster amplification was observed during flow cell generation (sequencing libraries).

B.5. Genomic DNA Isolation

For sequencing library construction and PCR reactions, genomic DNA was isolated from seed tissues of the test and control substances. First the seeds were decontaminated by vigorously agitating them by hand for 30 seconds with 0.05% (v/v) Tween-20, followed by a tap water rinse. The seeds were then vigorously agitated with 0.5% (w/v) NaOCl, allowed to stand for one minute at room temperature, and rinsed with tap water. The seeds were then vigorously agitated with 1% (v/v) HCl, allowed to stand for one minute at room temperature, and rinsed with tap water. The 1% (v/v) HCl rinse was repeated one time, and then the seeds were rinsed with distilled water and air dried. The dried seeds were ground to a fine powder in a GenoGrinder® SPEX 2010 homogenizer (SPEX SamplePrep, LLC). Genomic DNA was extracted using a Qiagen® DNeasy® Plant Mini Kit (Qiagen GmbH), according to manufacturer's instructions. All extracted DNA was stored in a 4°C refrigerator or a -20°C freezer.

B.6. DNA Quantification

Plasmid DNA and extracted genomic DNA were quantified using a Nanodrop™ Spectrophotometer (Thermo Scientific) or a Qubit Fluorometer (Invitrogen).

B.7. Paired End Library Preparation

DNA from the test, control, and reference samples, including transformation plasmids, were submitted to Nucleic Acid Technologies (Bayer) for library preparation and whole genome sequencing.

The DNA samples were fragmented by sonication using a Covaris sonicator (Covaris). Following fragmentation, the test, control, and reference DNA were made into sequencing libraries with TruSeq DNA UD Indexes using a KAPA Hyper library preparation kit following the manufactures instructions with the following exceptions. 1) The sample cleanup steps utilized BioChain magnetic beads. 2) The library amplification step utilized a PCR program with the following cycling conditions: 1 cycle at 98°C for 30 seconds; 5 cycles at 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and 1 cycle at 72°C for 5 minutes. 3) Library size selection (~ 500 bp) was performed with a 1.5% agarose gel cassette run on a BluePippin Size Selection system (Sage Science).

After preparation, libraries were analyzed using a Fragment Analyzer QC (Advanced Analytical Technologies, Inc.) according to the manufacturer's instructions. All purified library DNA was stored in a -20°C freezer.

B.8. Next-Generation Sequencing

The library samples described above were sequenced by Bayer's Nucleic Acid Technologies using Illumina short read technology that produced short sequence reads (~150 bp long). Sufficient numbers of these sequence reads were obtained to comprehensively cover the entire genomes of the test samples, conventional controls, and reference substances (transformation vectors) (Cade et al., 2018; Kovalic et al., 2012). More details can be found in Appendix K; Table KI. Furthermore, a transformation plasmid spike was sequenced to >75× to assess method sensitivity through modelling of $1/10^{th}$ and one full genome equivalent plasmid spike.

B.9. Read Mapping and Junction Identification

High-throughput sequence reads were captured by aligning to the PV-ZMIR522223 transformation plasmid sequence using the read alignment software Bowtie2 version 2.3.4.3 (Langmead and Salzberg, 2012) in order to collect all reads that were sourced from the plasmid as well as reads with sequences representing integration points (parameters: bowtie2 --very-fast-local -X 1000 --gbar 10 --dovetail --no-unal). No additional read quality filtering was applied. The high-throughput sequence reads from F4 and conventional LH244 were also aligned to the PV-ZMOO513642 transformation plasmid sequence utilizing the same steps and software outlined above. All software versions were documented in the archived data package and the software versions which were used in this study have been archived.

B.9.1. Mapping and Junction Detection

Captured reads from all test and control samples were mapped to the complete PV-ZMIR522223 transformation plasmid sequence and captured reads from F4 and conventional LH244 were mapped to the complete PV-ZMOO513642 plasmid sequence in order to detect junction sequences using the Bowtie2 (version 2.3.4.3) program. Reads with partial matches to the transformation plasmid of at least 30 bases and 96.6% or greater identity were also identified as potential junction sequences (Kovalic et al., 2012).

B.9.2. Effective Sequencing Depth Determination

A single copy locus from the native plant genome (*Zea mays*) *pyruvate decarboxylase* (*pdc3*, GenBank accession AF370006.2) was used to estimate the sequence depth coverage. All reads with at least 30 bases match and 96.6% identity were considered as reads sourced from this locus and used to calculate the final sequencing depth. Further details, mapping figures, and depth metrics can be found in Appendix K.

B.9.3. Summary NGS Data for the Control and Test Substances

For each sample, the raw data produced are presented in terms of total nucleotide number. The reads generated for this study were a maximum of 151bp in length. Estimated sequencing depth is provided in Appendix K, Table K1. Effective depth of coverage for this study was determined by mapping and alignment of all raw data to a single copy locus within the *Zea mays* genome (AF370006.2) and is shown for F4 in Figure IV-5, and all subsequent samples in Appendix K Figures K8-K14. Appendix Table K1 provides a comparison of total generated data, hypothetical coverage, and effective median coverage for all samples.

A graph of positional depths across samples relative to the transformation construct are shown in panel 3 of Figure IV-6 and Appendix K Figures K1-K6. Coverage statistics derived from the T-DNA regions of these samples are further provided in Appendix K Table K2.

B.9.4. Positive Control

To produce the positive control sample for sequencing, a plasmid DNA library was created for PV-ZMIR522223 and PV-ZMOO513642 as described in Section B.7 and was loaded at approximately ~0.04 % and ~0.02 %, respectively, of the total sequenced libraries. The collected data were sampled to represent a single genome equivalent dataset and a $1/10^{th}$ genome equivalent dataset.

B.10. PCR and DNA Sequence Analyses to Examine the Insert and Flanking Sequences in MON 95379

Overlapping PCR products, denoted as Product A and Product B were generated that span the insert and adjacent 5′ and 3′ flanking DNA sequences in MON 95379. For each fragment generation, experimental conditions were chosen to successfully produce on-target amplifications. These products were analyzed to determine the nucleotide sequence of the insert in MON 95379, as well as that of the DNA flanking the 5′ and 3′ ends of the insert, as depicted in Figure IV-9.

The PCR analyses for Product A was conducted using approximately 50 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.2 µM of each primer, 0.2 mM of each dNTP, and 1.25 units/reaction of PrimeSTAR GXL Polymerase (TaKaRa Bio Inc.).

The PCR amplification of Product A was performed under the following cycling conditions: 1 cycle at 98°C for 1 minute; 35 cycles at 98°C for 10 seconds, 68°C for 10 minutes.

The PCR amplification of Product B was performed under the following cycling conditions: 1 cycle at 98°C for 30 seconds; 35 cycles at 98°C for 10 seconds, 64°C for 20 seconds, 72°C for 2.5 minutes and 1 cycle at 72°C for 5 minutes.

Aliquots of each PCR product were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected sizes. Each PCR product was purified with Agencourt AMPure XP (Beckman Coulter Life Sciences). Approximately 200 ng of purified amplicon was submitted to Bayer's Nucleic Acid Technologies for library preparation and MiSeq sequencing.

A consensus sequence was generated by compiling captured reads from MiSeq sequencing of the overlapping PCR products. This consensus sequence was aligned to the PV-ZMIR522223 sequence to determine the integrity and organization of the integrated DNA and the 5′ and 3′ insert-to-flank DNA junctions in MON 95379.

B.11. PCR and DNA Sequence Analyses to Examine the Integrity of the DNA Insertion Site in MON 95379

To examine the MON 95379 T-DNA insertion site in LH244 conventional maize, PCR and sequence analyses were performed on genomic DNA from the LH244 conventional control as depicted in Figure IV-10.

The primers used in this analysis were designed from the DNA sequences flanking the insert in MON 95379. A forward primer specific to the DNA sequence flanking the 5′ end of the insert was paired with a reverse complement primer specific to the DNA sequence flanking the 3′ end of the insert.

The PCR reactions were conducted using approximately 50 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.2 µM of each primer, 0.2 mM of each dNTP, and 1.25 units/reaction of PrimeSTAR GXL Polymerase. The PCR amplification was performed under the following cycling conditions: 1 cycle at 98°C for 1 minute; 35 cycles at 98°C for 10 seconds, 68°C for 2.5 minutes.

Aliquots of each PCR product were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected sizes. Each PCR product was purified with Agencourt AMPure XP (Beckman Coulter Life Sciences). Approximately 200 ng of purified amplicon was submitted to Bayer's Nucleic Acid Technologies for library preparation and MiSeq sequencing.

A consensus sequence was generated by compiling sequences from MiSeq sequencing reactions of the verified PCR product. This consensus sequence was aligned to the 5′ and 3′ sequences flanking the MON 95379 insert to determine the integrity and any rearrangement of the insertion site.

B.12. Protocol Amendments and Deviations

During the study, changes were made to the original protocol. The changes were documented as protocol amendments and are summarized below. These changes positively impacted the quality and integrity of the study data and conclusions.

1. The protocol was amended to include PV-ZMOO513642 as a reference material.
2. The protocol was amended to clarify the algorithms used to analyze the next generation sequencing data.
3. The protocol was also amended to update the breeding generations to be tested.

B.13. Rejected Data

Rejected data were assessed to have no effect on the quality, integrity, or conclusions of the study and are retained in the study package (Appendix L).

References for Appendix B

Cade, R., K. Burgin, K. Schilling, T.-J. Lee, P. Ngam, N. Devitt and D. Fajardo. 2018. Evaluation of whole genome sequencing and an insertion site characterization method for molecular characterization of GM maize. Journal of Regulatory Science 6:1-14.

Kovalic, D., C. Garnaat, L. Guo, Y. Yan, J. Groat, A. Silvanovich, L. Ralston, M. Huang, Q. Tian, A. Christian, N. Cheikh, J. Hjelle, S. Padgette and G. Bannon. 2012. The use of next generation sequencing and junction sequence analysis bioinformatics to achieve molecular characterization of crops improved through modern biotechnology. The Plant Genome 5:149-163.

Langmead, B. and S.L. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9:357-359.

Appendix C: Characterization of Cry1B.868 Protein Produced in MON 95379

C.1. Characterization of the Cry1B.868 Protein

C.1.1. Cry1B.868 Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties and confirmation of the safety of the introduced protein(s). For the safety data generated using the *Bt*-produced Cry1B.868 protein to be applied to the MON 95379-produced Cry1B.868 protein (plant-produced Cry1B.868), the equivalence of the plant- and *Bt*-produced proteins must first be demonstrated. To assess the equivalence between the MON 95379-produced and *Bt*-produced Cry1B.868 proteins, a small quantity of the MON 95379-produced Cry1B.868 protein was purified from MON 95379 grain. The MON 95379-produced Cry1B.868 protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 95379-produced and *Bt*-produced Cry1B.868 proteins was assessed using a panel of analytical tests; as shown in Table C-1. Taken together, these data provide a detailed characterization of the MON 95379-produced Cry1B.868 protein and establish the equivalence of the MON 95379-produced and *Bt*-produced Cry1B.868 proteins. Based on this established equivalence, conclusions derived from digestibility, heat susceptibility, acute oral toxicology study and non-target organism studies conducted with *Bt*-produced Cry1B.868 protein are applicable to MON 95379-produced Cry1B.868 protein.

Table C-1. Summary of MON 95379 Cry1B.868 Protein Identity and Equivalence

Analytical Test	Assessment	Analytical Test Outcome
N-terminal sequence	Identity	The expected N-terminal sequence for MON 95379-produced Cry1B.868 protein was observed by Nano LC-MS/MS[1]
Nano LC-MS/MS[1]	Identity	Nano LC-MS/MS[1] analysis of trypsin or Asp-N digested peptides from MON 95379-produced Cry1B.868 protein yielded peptide masses consistent with expected peptide masses from the theoretical trypsin or Asp-N digest of the amino acid sequence
Western blot analysis	Identity and Equivalence	MON 95379-produced Cry1B.868 protein identity was confirmed using a western blot probed with antibodies specific for Cry1B.868 protein Immunoreactive properties of the MON 95379-produced Cry1B.868 and the *Bt*-produced Cry1B.868 proteins were shown to be equivalent
Apparent molecular weight (MW)	Equivalence	Electrophoretic mobility and apparent molecular weight of the MON 95379-produced Cry1B.868 and the *Bt*-produced Cry1B.868 proteins were shown to be equivalent
Glycosylation analysis	Equivalence	Glycosylation status of MON 95379-produced Cry1B.868 and *Bt*-produced Cry1B.868 proteins were shown to be equivalent
Functional activity	Equivalence	Functional activity of the MON 95379-produced Cry1B.868 and the *Bt*-produced Cry1B.868 proteins were shown to be equivalent by insect bioassay

[1] Nano LC-MS/MS = Nanoscale liquid chromatography-tandem mass spectrometry

The details of the materials and methods for the panel of analytical tests used to evaluate and compare the properties of the MON 95379-produced Cry1B.868 and *Bt*-produced Cry1B.868 proteins are described at the end of Appendix C. A summary of the data obtained to support a conclusion of protein equivalence is provided below.

C.1.2. Results of the N-Terminal Sequencing Analysis

The identification of N-terminal sequence was conducted by LC-MS/MS analysis of peptide fragments produced through enzymatic digestion of the MON 95379-produced Cry1B.868 protein with Asp-N. The expected N-terminal sequence for the Cry1B.868 protein deduced from the *Cry1B.868* gene present in maize of MON 95379 was observed, except that the N-terminal methionine was cleaved *in vivo* from the MON 95379-produced Cry1B.868 by methionine aminopeptidase (see Experimental Sequence, Figure C-1). The cleavage of the N-terminal methionine from proteins *in vivo* by methionine aminopeptidase is common in many organisms (Bradshaw et al., 1998; Wang et al., 2016). The N-terminal sequence for MON 95379-produced Cry1B.868 protein was consistent with the N-terminal sequence for the *Bt*-produced Cry1B.868 protein observed by LC-MS/MS (Figure C-1). An additional minor form of the MON 95379-produced Cry1B.868 protein with the N-terminal methionine was also observed. Hence, the sequence information confirms the identity of the Cry1B.868 protein isolated from the grain of MON 95379.

Amino Acids Residue # from the N-terminus	→	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Bt-produced Cry1B.868 sequence	→	-	T	S	N	R	K	N	E	N	E	I	I	N	A	L	S
			|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Expected Cry1B.868 Sequence	→	M	T	S	N	R	K	N	E	N	E	I	I	N	A	L	S
			|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
MON 95379 Experimental Sequence	→	-	T	S	N	R	K	N	E	N	E	I	I	N	A	L	S

Figure C-1. N-Terminal Sequence of the MON 95379-Produced Cry1B.868 Protein

The experimental sequence obtained from the MON 95379-produced Cry1B.868 was compared to the expected sequence deduced from the *cry1B.868* gene present in MON 95379. *Bt*-produced Cry1B.868 protein sequence above was derived from the reference substance Certificate of Analysis (COA) (lot 7565). The single-letter International Union of Pure and Applied Chemistry - International Union of Biochemistry (IUPAC-IUB) amino acid code is M, methionine; T, Threonine; S, Serine; N, Asparagine; R, Arginine; K, Lysine; E, Glutamic acid; I, Isoleucine; A, Alanine; L, Leucine.

C.1.3. Results of Mass Fingerprint Analysis

Peptide mass fingerprint analysis is a standard technique used for confirming the identity of proteins. The identity of the MON 95379-produced Cry1B.868 protein was confirmed by LC-MS/MS analysis of peptide fragments produced through enzymatic digestion of the MON 95379-produced Cry1B.868 protein.

There were 110 unique peptides identified that correspond to the masses expected to be produced by trypsin digestion of the MON 95379-produced Cry1B.868 protein (Table C-2). The identified peptides were used to assemble a sequence coverage map of the entire Cry1B.868 protein (Figure C-2, Panel A). The experimentally determined coverage of the MON 95379-produced Cry1B.868 protein was 91% (Figure C-2, Panel A, 1088 out of 1199 amino acids). This analysis further confirms the identity of MON 95379-produced Cry1B.868 protein.

There were 145 unique peptides identified that correspond to the masses expected to be produced by trypsin digestion of the *Bt*-produced Cry1B.868 protein (Table C-3) by LC-MS/MS analysis during the protein characterization. The identified peptides were used to assemble a coverage map of the entire Cry1B.868 protein (Figure C-2, Panel B). The experimentally determined coverage of the *Bt*-produced Cry1B.868 protein was 96% (Figure C-2, Panel B, 1156 out of 1199 amino acids).

Table C-2. Summary of the Tryptic Masses Identified for the MON 95379-Produced Cry1B.868 Using LC-MS/MS[1]

Experimental Mass[2]	Calculated Mass[3]	Diff[4]	Fragment[5]	Sequence[6]
3136.5591	3136.5571	0.0020	6 - 34	KNEN...TDAR
3008.4627	3008.4621	0.0006	7 - 34	NENE...TDAR
3261.5882	3261.5823	0.0059	35 - 65	IEDS...IAGR
2935.6012	2935.6000	0.0012	66 - 92	ILGV...LWPR
2136.1137	2136.1116	0.0021	93 - 109	GRDP...QLIR
1922.9901	1922.9890	0.0011	95 - 109	DPWE...QLIR
974.4775	974.4781	-0.0006	110 - 117	QQVTENTR
1601.8130	1601.8121	0.0009	110 - 123	QQVT...ALAR
645.3443	645.3446	-0.0003	118 - 123	DTALAR
990.5252	990.5247	0.0005	124 - 132	LQGL...NSFR
1650.7638	1650.7638	0.0000	133 - 145	AYQQ...LENR
2107.9605	2107.9559	0.0046	133 - 149	AYQQ...DDAR
2800.4884	2800.4873	0.0011	152 - 175	SVLY...FAIR
2518.4144	2518.4093	0.0051	176 - 197	NQEV...LLLR
1983.9540	1983.9538	0.0002	198 - 215	DASL...EIQR
629.2809	629.2809	0.0000	216 - 219	YYER
502.2752	502.2751	0.0001	220 - 223	QVEK
1249.6222	1249.6203	0.0019	234 - 243	WYNT...NNLR
1032.4997	1032.4988	0.0009	244 - 252	GTNA...SWLR
726.3454	726.3449	0.0005	253 - 257	YNQFR
2263.2201	2263.2212	-0.0011	258 - 277	RDLT...YDTR
2107.1237	2107.1201	0.0036	259 - 277	DLTL...YDTR
1379.6874	1379.6867	0.0007	278 - 289	VYPM...QLTR
1062.5349	1062.5346	0.0003	290 - 298	EIYT...PIGR
5243.6491	5243.6469	0.0022	299 - 346	TNAP...VLSR
1840.8100	1840.8104	-0.0004	347 - 360	WSNT...VGHR
503.2703	503.2703	0.0000	361 - 364	LESR
2619.2933	2619.2889	0.0044	368 - 392	GSLS...FTSR
551.2703	551.2704	-0.0001	393 - 396	DVYR
2888.5213	2888.5185	0.0028	393 - 418	DVYR...PWAR
2355.2594	2355.2587	0.0007	397 - 418	TESF...PWAR
621.3022	621.3023	-0.0001	419 - 422	FNWR
1415.7410	1415.7422	-0.0012	419 - 429	FNWR...NSLR
812.4502	812.4504	-0.0002	423 - 429	NPLNSLR
4407.0833	4407.0819	0.0014	430 - 468	GSLL...YSHR
601.3545	601.3547	-0.0002	469 - 473	LSNIR
872.5075	872.5080	-0.0005	474 - 481	LISGNTLR

Experimental Mass[2]	Calculated Mass[3]	Diff[4]	Fragment[5]	Sequence[6]
1115.5512	1115.5512	0.0000	482 - 490	APVY...WTHR
447.2077	447.2077	0.0000	491 - 494	SADR
2158.1219	2158.1230	-0.0011	491 - 510	SADR...PLVK
1728.9273	1728.9258	0.0015	495 - 510	TNTI...PLVK
2089.1224	2089.1168	0.0056	495 - 513	TNTI...KGFR
2349.2223	2349.2230	-0.0007	511 - 533	GFRV...DILR
1989.0345	1989.0320	0.0025	514 - 533	VWGG...DILR
2145.1336	2145.1331	0.0005	514 - 534	VWGG...ILRR
2405.2430	2405.2452	-0.0022	534 - 554	RNTF...ITQR
2249.1461	2249.1441	0.0020	535 - 554	NTFG...ITQR
582.2760	582.2762	-0.0002	561 - 565	YASSR
2325.2765	2325.2726	0.0039	569 - 592	VIVL...PLQK
1249.5978	1249.5972	0.0006	593 - 603	TMEI...LTSR
1379.6156	1379.6146	0.0010	607 - 617	YTDF...FSFR
2890.4515	2890.4600	-0.0085	618 - 645	ANPD...YIDK
4906.4687	4906.4651	0.0036	618 - 663	ANPD...DLER
2034.0166	2034.0157	0.0009	646 - 663	IEII...DLER
1947.0454	1947.0425	0.0029	664 - 681	AQKA...IGLK
1619.8520	1619.8519	0.0001	667 - 681	AVNE...IGLK
732.4017	732.4017	0.0000	709 - 714	KELSEK
604.3070	604.3068	0.0002	710 - 714	ELSEK
774.3987	774.3984	0.0003	720 - 725	RLSDER
618.2979	618.2973	0.0006	721 - 725	LSDER
1715.8592	1715.8591	0.0001	721 - 734	LSDE...PNFR
1115.5730	1115.5723	0.0007	726 - 734	NLLQ...PNFR
458.2602	458.2601	0.0001	735 - 738	GINR
530.2812	530.2813	-0.0001	739 - 742	QLDR
1551.7420	1551.7417	0.0003	746 - 760	GSTD...DVFK
4150.9422	4150.9245	0.0177	746 - 781	GSTD...LYQK
590.2911	590.2911	0.0000	782 - 786	IDESK
509.2598	509.2598	0.0000	789 - 792	AYTR
578.3175	578.3176	-0.0001	793 - 796	YQLR
1825.9111	1825.9098	0.0013	797 - 811	GYIE...YLIR
494.2489	494.2489	0.0000	812 - 815	YNAK
2819.4554	2819.4606	-0.0052	812 - 838	YNAK...PIGK
2343.2223	2343.2223	0.0000	816 - 838	HETV...PIGK
1024.5414	1024.5414	0.0000	869 - 877	IKTQ...GHAR
783.3622	783.3624	-0.0002	871 - 877	TQDGHAR
2097.1480	2097.1470	0.0010	878 - 896	LGNL...ALAR

Experimental Mass[2]	Calculated Mass[3]	Diff[4]	Fragment[5]	Sequence[6]
502.2863	502.2863	0.0000	899 - 902	RAEK
474.2802	474.2802	0.0000	900 - 903	AEKK
1550.7981	1550.7980	0.0001	909 - 920	EKLE...IVYK
1293.6601	1293.6605	-0.0004	911 - 920	LEWE...IVYK
1969.9390	1969.9381	0.0009	921 - 937	EAKE...QYDR
1641.7639	1641.7635	0.0004	924 - 937	ESVD...QYDR
1610.8088	1610.8086	0.0002	938 - 952	LQAD...AADK
1766.9090	1766.9097	-0.0007	938 - 953	LQAD...ADKR
766.4563	766.4562	0.0001	953 - 958	RVHSIR
610.3547	610.3551	-0.0004	954 - 958	VHSIR
2615.3490	2615.3482	0.0008	959 - 982	EAYL...LEGR
1302.6615	1302.6608	0.0007	983 - 993	IFTA...YDAR
1756.9508	1756.9512	-0.0004	983 - 997	IFTA...NVIK
472.3006	472.3009	-0.0003	994 - 997	NVIK
2078.9832	2078.9844	-0.0012	994 - 1011	NVIK...WNVK
1624.6944	1624.6940	0.0004	998 - 1011	NGDF...WNVK
1432.6460	1432.6443	0.0017	1012 - 1023	GHVD...NNHR
1955.0016	1955.0000	0.0016	1024 - 1040	SVLV...QEVR
620.3647	620.3646	0.0001	1046 - 1050	GYILR
580.3217	580.3221	-0.0004	1051 - 1055	VTAYK
2969.3562	2969.3600	-0.0038	1051 - 1076	VTAY...DELK
2407.0481	2407.0485	-0.0004	1056 - 1076	EGYG...DELK
6460.6970	6460.6802	0.0168	1056 - 1110	EGYG...YTSR
4071.6560	4071.6422	0.0138	1077 - 1110	FSNC...YTSR
2742.1708	2742.1681	0.0027	1111 - 1135	NRGY...YEEK
2472.0260	2472.0241	0.0019	1113 - 1135	GYDG...YEEK
3135.3217	3135.3217	0.0000	1113 - 1141	GYDG...TDGR
681.3079	681.3082	-0.0003	1136 - 1141	AYTDGR
837.4118	837.4093	0.0025	1136 - 1142	AYTDGRR
2730.2378	2730.2344	0.0034	1142 - 1165	RDNP...YVTK
2574.1331	2574.1333	-0.0002	1143 - 1165	DNPC...YVTK
1600.7776	1600.7773	0.0003	1151 - 1165	GYGD...YVTK
1269.5754	1269.5765	-0.0011	1166 - 1175	ELEY...ETDK
4001.9276	4001.9271	0.0005	1166 - 1199	ELEY...LMEE

[1]All imported values were rounded to 4 decimal places.
[2]Only experimental masses that matched calculated masses with the highest scores are listed in the table.
[3] The calculated mass is the exact molecular mass calculated from the matched peptide sequence.
[4] The calculated difference = experimental mass – calculated mass.
[5] Position refers to amino acid residues within the predicted MON 95379-produced Cry1B.868 sequence as depicted in Figure C-2, Panel A.
[6] For peptide matches greater than nine amino acids in length, the first 4 residues and last 4 residues are show separated by three dots (...).

Table C-3. Summary of the Tryptic Masses Identified for the *Bt*-Produced Cry1B.868 Using LC-MS/MS[1]

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
3594.7858	3594.7808	0.0050	2 - 34	TSNR...TDAR
3136.5591	3136.5571	0.0020	6 - 34	KNEN...TDAR
3008.4640	3008.4621	0.0019	7 - 34	NENE...TDAR
3260.6014	3260.5983	0.0031	35 - 65	IEDS...IAGR
6178.1936	6178.1878	0.0058	35 - 92	IEDS...LWPR
2935.6029	2935.6000	0.0029	66 - 92	ILGV...LWPR
2136.1155	2136.1116	0.0039	93 - 109	GRDP...QLIR
3092.5798	3092.5792	0.0006	93 - 117	GRDP...ENTR
1922.9911	1922.9890	0.0021	95 - 109	DPWE...QLIR
974.4786	974.4781	0.0005	110 - 117	QQVTENTR
1601.8121	1601.8121	0.0000	110 - 123	QQVT...ALAR
645.3440	645.3446	-0.0006	118 - 123	DTALAR
990.5245	990.5247	-0.0002	124 - 132	LQGL...NSFR
1650.7633	1650.7638	-0.0005	133 - 145	AYQQ...LENR
2107.9515	2107.9559	-0.0044	133 - 149	AYQQ...DDAR
2365.1092	2365.1047	0.0045	133 - 151	AYQQ...ARTR
3057.6331	3057.6361	-0.0030	150 - 175	TRSV...FAIR
2800.4818	2800.4873	-0.0055	152 - 175	SVLY...FAIR
7266.8344	7266.8293	0.0051	152 - 215	SVLY...EIQR
2518.4161	2518.4093	0.0068	176 - 197	NQEV...LLLR
4484.3640	4484.3526	0.0114	176 - 215	NQEV...EIQR
1983.9531	1983.9538	-0.0007	198 - 215	DASL...EIQR
1113.5454	1113.5454	0.0000	216 - 223	YYERQVEK
1319.5563	1319.5564	-0.0001	224 - 233	TREY...YCAR
1062.4082	1062.4076	0.0006	226 - 233	EYSDYCAR
1249.6207	1249.6203	0.0004	234 - 243	WYNT...NNLR
2264.1086	2264.1086	0.0000	234 - 252	WYNT...SWLR
1032.4995	1032.4988	0.0007	244 - 252	GTNA...SWLR
1896.9329	1896.9343	-0.0014	244 - 258	GTNA...QFRR
882.4483	882.4460	0.0023	253 - 258	YNQFRR
2263.2196	2263.2212	-0.0016	258 - 277	RDLT...YDTR
2107.1212	2107.1201	0.0011	259 - 277	DLTL...YDTR
3468.7981	3468.7963	0.0018	259 - 289	DLTL...QLTR
4513.3151	4513.3203	-0.0052	259 - 298	DLTL...PIGR
1379.6881	1379.6867	0.0014	278 - 289	VYPM...QLTR
1062.5338	1062.5346	-0.0008	290 - 298	EIYT...PIGR
5243.6491	5243.6469	0.0022	299 - 346	TNAP...VLSR

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
7066.4556	7066.4467	0.0089	299 - 360	TNAP...VGHR
1840.8100	1840.8104	-0.0004	347 - 360	WSNT...VGHR
2326.0712	2326.0701	0.0011	347 - 364	WSNT...LESR
2989.5168	2989.5217	-0.0049	365 - 392	TIRG...FTSR
2619.2933	2619.2889	0.0044	368 - 392	GSLS...FTSR
3152.5479	3152.5487	-0.0008	368 - 396	GSLS...DVYR
5489.7927	5489.7968	-0.0041	368 - 418	GSLS...PWAR
2888.5197	2888.5185	0.0012	393 - 418	DVYR...PWAR
2355.2601	2355.2587	0.0014	397 - 418	TESF...PWAR
1415.7423	1415.7422	0.0001	419 - 429	FNWR...NSLR
5804.8251	5804.8135	0.0116	419 - 468	FNWR...YSHR
812.4504	812.4504	0.0000	423 - 429	NPLNSLR
5201.5275	5201.5218	0.0057	423 - 468	NPLN...YSHR
4407.0847	4407.0819	0.0028	430 - 468	GSLL...YSHR
1455.8525	1455.8521	0.0004	469 - 481	LSNI...NTLR
2553.3958	2553.3928	0.0030	469 - 490	LSNI...WTHR
872.5078	872.5080	-0.0002	474 - 481	LISGNTLR
1970.0517	1970.0486	0.0031	474 - 490	LISG...WTHR
1115.5515	1115.5512	0.0003	482 - 490	APVY...WTHR
2158.1246	2158.1230	0.0016	491 - 510	SADR...PLVK
1728.9264	1728.9258	0.0006	495 - 510	TNTI...PLVK
2089.1186	2089.1168	0.0018	495 - 513	TNTI...KGFR
4060.1354	4060.1382	-0.0028	495 - 533	TNTI...DILR
2349.2274	2349.2230	0.0044	511 - 533	GFRV...DILR
2505.3200	2505.3241	-0.0041	511 - 534	GFRV...ILRR
1989.0338	1989.0320	0.0018	514 - 533	VWGG...DILR
2145.1384	2145.1331	0.0053	514 - 534	VWGG...ILRR
2405.2503	2405.2452	0.0051	534 - 554	RNTF...ITQR
2249.1415	2249.1441	-0.0026	535 - 554	NTFG...ITQR
2325.2740	2325.2726	0.0014	569 - 592	VIVL...PLQK
1249.5969	1249.5972	-0.0003	593 - 603	TMEI...LTSR
1783.8307	1783.8318	-0.0011	604 - 617	TFRY...FSFR
1379.6152	1379.6146	0.0006	607 - 617	YTDF...FSFR
2890.4637	2890.4600	0.0037	618 - 645	ANPD...YIDK
4906.4672	4906.4651	0.0021	618 - 663	ANPD...DLER
5233.6603	5233.6557	0.0046	618 - 666	ANPD...RAQK
1947.0437	1947.0425	0.0012	664 - 681	AQKA...IGLK
1619.8526	1619.8519	0.0007	667 - 681	AVNE...IGLK
3243.4239	3243.4224	0.0015	682 - 708	TDVT...LDEK

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
3371.5164	3371.5174	-0.0010	682 - 709	TDVT...DEKK
3957.8241	3957.8136	0.0105	682 - 714	TDVT...LSEK
732.4015	732.4017	-0.0002	709 - 714	KELSEK
831.4702	831.4702	0.0000	710 - 716	ELSEKVK
774.3982	774.3984	-0.0002	720 - 725	RLSDER
1871.9588	1871.9602	-0.0014	720 - 734	RLSD...PNFR
1715.8591	1715.8591	0.0000	721 - 734	LSDE...PNFR
2156.1103	2156.1086	0.0017	721 - 738	LSDE...GINR
1115.5726	1115.5723	0.0003	726 - 734	NLLQ...PNFR
1555.8231	1555.8219	0.0012	726 - 738	NLLQ...GINR
970.5311	970.5308	0.0003	735 - 742	GINRQLDR
929.4830	929.4831	-0.0001	739 - 745	QLDRGWR
4549.1413	4549.1424	-0.0011	743 - 781	GWRG...LYQK
1551.7441	1551.7417	0.0024	746 - 760	GSTD...DVFK
4149.9435	4149.9405	0.0030	746 - 781	GSTD...LYQK
2616.2074	2616.2094	-0.0020	761 - 781	ENYV...LYQK
831.4697	831.4702	-0.0005	782 - 788	IDESKLK
1322.7212	1322.7194	0.0018	782 - 792	IDES...AYTR
750.4382	750.4388	-0.0006	787 - 792	LKAYTR
1069.5662	1069.5668	-0.0006	789 - 796	AYTRYQLR
1825.9099	1825.9098	0.0001	797 - 811	GYIE...YLIR
2302.1537	2302.1481	0.0056	797 - 815	GYIE...YNAK
4627.3601	4627.3598	0.0003	797 - 838	GYIE...PIGK
2819.4571	2819.4606	-0.0035	812 - 838	YNAK...PIGK
2343.2218	2343.2223	-0.0005	816 - 838	HETV...PIGK
3520.6328	3520.6293	0.0035	839 - 868	CAHH...VIFK
1024.5414	1024.5414	0.0000	869 - 877	IKTQ...GHAR
783.3623	783.3624	-0.0001	871 - 877	TQDGHAR
2862.5031	2862.4988	0.0043	871 - 896	TQDG...ALAR
2097.1474	2097.1470	0.0004	878 - 896	LGNL...ALAR
1706.8983	1706.8991	-0.0008	908 - 920	REKL...IVYK
1550.8030	1550.7980	0.0050	909 - 920	EKLE...IVYK
1878.9717	1878.9727	-0.0010	909 - 923	EKLE...KEAK
1293.6614	1293.6605	0.0009	911 - 920	LEWE...IVYK
1621.8354	1621.8351	0.0003	911 - 923	LEWE...KEAK
1969.9380	1969.9381	-0.0001	921 - 937	EAKE...QYDR
1641.7602	1641.7635	-0.0033	924 - 937	ESVD...QYDR
3390.6601	3390.6626	-0.0025	924 - 953	ESVD...ADKR
1610.8082	1610.8086	-0.0004	938 - 952	LQAD...AADK

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
1766.9128	1766.9097	0.0031	938 - 953	LQAD...ADKR
766.4567	766.4562	0.0005	953 - 958	RVHSIR
4492.3474	4492.3430	0.0044	954 - 993	VHSI...YDAR
2615.3494	2615.3482	0.0012	959 - 982	EAYL...LEGR
3900.0148	3899.9985	0.0163	959 - 993	EAYL...YDAR
1302.6617	1302.6608	0.0009	983 - 993	IFTA...YDAR
1756.9504	1756.9512	-0.0008	983 - 997	IFTA...NVIK
1432.6447	1432.6443	0.0004	1012 - 1023	GHVD...NNHR
3369.6337	3369.6338	-0.0001	1012 - 1040	GHVD...QEVR
1954.9977	1955.0000	-0.0023	1024 - 1040	SVLV...QEVR
2524.2738	2524.2744	-0.0006	1024 - 1045	SVLV...CPGR
1189.6406	1189.6390	0.0016	1041 - 1050	VCPG...YILR
1182.6760	1182.6761	-0.0001	1046 - 1055	GYIL...TAYK
2968.3764	2968.3760	0.0004	1051 - 1076	VTAY...DELK
4069.6770	4069.6742	0.0028	1077 - 1110	FSNC...YTSR
4339.8198	4339.8182	0.0016	1077 - 1112	FSNC...SRNR
2742.1699	2742.1681	0.0018	1111 - 1135	NRGY...YEEK
3405.4638	3405.4657	-0.0019	1111 - 1141	NRGY...TDGR
2472.0208	2472.0241	-0.0033	1113 - 1135	GYDG...YEEK
3135.3206	3135.3217	-0.0011	1113 - 1141	GYDG...TDGR
3291.4220	3291.4228	-0.0008	1113 - 1142	GYDG...DGRR
681.3083	681.3082	0.0001	1136 - 1141	AYTDGR
837.4092	837.4093	-0.0001	1136 - 1142	AYTDGRR
1146.4839	1146.4836	0.0003	1142 - 1150	RDNP...ESNR
2729.2520	2729.2504	0.0016	1142 - 1165	RDNP...YVTK
990.3831	990.3825	0.0006	1143 - 1150	DNPCESNR
2573.1489	2573.1493	-0.0004	1143 - 1165	DNPC...YVTK
1600.7778	1600.7773	0.0005	1151 - 1165	GYGD...YVTK
1269.5777	1269.5765	0.0012	1166 - 1175	ELEY...ETDK
4001.9221	4001.9271	-0.0050	1166 - 1199	ELEY...LMEE

[1] All imported values were rounded to 4 decimal places.
[2] Only experimental masses that matched calculated masses with the highest scores are listed in table.
[3] The calculated mass is the exact molecular mass calculated from the matched peptide sequence.
[4] The calculated difference = experimental mass – calculated mass.
[5] Position refers to amino acid residues within the predicted *Bt*-produced Cry1B.868 sequence as depicted in Figure C-2, Panel B.
[6] For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are shown separated by dots (…).

(A)

```
   1 MTSNRKNENE IINALSIPAV SNHSAQMNLS TDARIEDSLC IAEGNNIDPF
  51 VSASTVQTGI NIAGRILGVL GVPFAGQIAS FYSFLVGELW PRGRDPWEIF
 101 LEHVEQLIRQ QVTENTRDTA LARLQGLGNS FRAYQQSLED WLENRDDART
 151 RSVLYTQYIA LELDFLNAMP LFAIRNQEVP LLMVYAQAAN LHLLLLRDAS
 201 LFGSEFGLTS QEIQRYYERQ VEKTREYSDY CARWYNTGLN NLRGTNAESW
 251 LRYNQFRRDL TLGVLDLVAL FPSYDTRVYP MNTSAQLTRE IYTDPIGRTN
 301 APSGFASTNW FNNNAPSFSA IEAAVIRPPH LLDFPEQLTI FSVLSRWSNT
 351 QYMNYWVGHR LESRTIRGSL STSTHGNTNT SINPVTLQFT SRDVYRTESF
 401 AGINILLTTP VNGVPWARFN WRNPLNSLRG SLLYTIGYTG VGTQLFDSET
 451 ELPPETTERP NYESYSHRLS NIRLISGNTL RAPVYSWTHR SADRTNTISS
 501 DSINQIPLVK GFRVWGGTSV ITGPGFTGGD ILRRNTFGDF VSLQVNINSP
 551 ITQRYRLRFR YASSRDARVI VLTGAASTGV GGQVSVNMPL QKTMEIGENL
 601 TSRTFRYTDF SNPFSFRANP DIIGISEQPL FGAGSISSGE LYIDKIEIIL
 651 ADATFEAESD LERAQKAVNE LFTSSNQIGL KTDVTDYHID QVSNLVECLS
 701 DEFCLDEKKE LSEKVKHAKR LSDERNLLQD PNFRGINRQL DRGWRGSTDI
 751 TIQGGDDVFK ENYVTLLGTF DECYPTYLYQ KIDESKLKAY TRYQLRGYIE
 801 DSQDLEIYLI RYNAKHETVN VPGTGSLWPL SAPSPIGKCA HHSHHFSLDI
 851 DVGCTDLNED LGVWVIFKIK TQDGHARLGN LEFLEEKPLV GEALARVKRA
 901 EKKWRDKREK LEWETNIVYK EAKESVDALF VNSQYDRLQA DTNIAMIHAA
 951 DKRVHSIREA YLPELSVIPG VNAAIFEELE GRIFTAFSLY DARNVIKNGD
1001 FNNGLSCWNV KGHVDVEEQN NHRSVLVVPE WEAEVSQEVR VCPGRGYILR
1051 VTAYKEGYGE GCVTIHEIEN NTDELKFSNC VEEEVYPNNT VTCNDYTATQ
1101 EEYEGTYTSR NRGYDGAYES NSSVPADYAS AYEEKAYTDG RRDNPCESNR
1151 GYGDYTPLPA GYVTKELEYF PETDKVWIEI GETEGTFIVD SVELLLMEE
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(B)

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   1 MTSNRKNENE IINALSIPAV SNHSAQMNLS TDARIEDSLC IAEGNNIDPF
  51 VSASTVQTGI NIAGRILGVL GVPFAGQIAS FYSFLVGELW PRGRDPWEIF
 101 LEHVEQLIRQ QVTENTRDTA LARLQGLGNS FRAYQQSLED WLENRDDART
 151 RSVLYTQYIA LELDFLNAMP LFAIRNQEVP LLMVYAQAAN LHLLLLRDAS
 201 LFGSEFGLTS QEIQRYYERQ VEKTREYSDY CARWYNTGLN NLRGTNAESW
 251 LRYNQFRRDL TLGVLDLVAL FPSYDTRVYP MNTSAQLTRE IYTDPIGRTN
 301 APSGFASTNW FNNNAPSFSA IEAAVIRPPH LLDFPEQLTI FSVLSRWSNT
 351 QYMNYWVGHR LESRTIRGSL STSTHGNTNT SINPVTLQFT SRDVYRTESF
 401 AGINILLTTP VNGVPWARFN WRNPLNSLRG SLLYTIGYTG VGTQLFDSET
 451 ELPPETTERP NYESYSHRLS NIRLISGNTL RAPVYSWTHR SADRTNTISS
 501 DSINQIPLVK GFRVWGGTSV ITGPGFTGGD ILRRNTFGDF VSLQVNINSP
 551 ITQRYRLRFR YASSRDARVI VLTGAASTGV GGQVSVNMPL QKTMEIGENL
 601 TSRTFRYTDF SNPFSFRANP DIIGISEQPL FGAGSISSGE LYIDKIEIIL
 651 ADATFEAESD LERAQKAVNE LFTSSNQIGL KTDVTDYHID QVSNLVECLS
 701 DEFCLDEKKE LSEKVKHAKR LSDERNLLQD PNFRGINRQL DRGWRGSTDI
 751 TIQGGDDVFK ENYVTLLGTF DECYPTYLYQ KIDESKLKAY TRYQLRGYIE
 801 DSQDLEIYLI RYNAKHETVN VPGTGSLWPL SAPSPIGKCA HHSHHFSLDI
 851 DVGCTDLNED LGVWVIFKIK TQDGHARLGN LEFLEEKPLV GEALARVKRA
 901 EKKWRDKREK LEWETNIVYK EAKESVDALF VNSQYDRLQA DTNIAMIHAA
 951 DKRVHSIREA YLPELSVIPG VNAAIFEELE GRIFTAFSLY DARNVIKNGD
1001 FNNGLSCWNV KGHVDVEEQN NHRSVLVVPE WEAEVSQEVR VCPGRGYILR
1051 VTAYKEGYGE GCVTIHEIEN NTDELKFSNC VEEEVYPNNT VTCNDYTATQ
1101 EEYEGTYTSR NRGYDGAYES NSSVPADYAS AYEEKAYTDG RRDNPCESNR
1151 GYGDYTPLPA GYVTKELEYF PETDKVWIEI GETEGTFIVD SVELLLMEE
```

Figure C-2. Peptide Map of the MON 95379-Produced Cry1B.868 and *Bt*-produced Cry1B.868 Proteins

(A). The amino acid sequence of the MON 95379-produced Cry1B.868 protein was deduced from the *cry1B.868* gene present in MON 95379. Boxed regions correspond to peptides that were identified from the MON 95379-produced Cry1B.868 protein sample using LC-MS/MS. In total, 91% coverage (1088 out of 1199 amino acids) of the expected protein sequence was covered by the identified peptides. Gray highlighted regions correspond to the receptor binding domain see Appendix L.

(B). The amino acid sequence of the *Bt*-produced Cry1B.868 protein was deduced from the *cry1B.868* gene that is contained on the expression plasmid pMON236906. Boxed regions correspond to peptides that were identified from the *Bt*-produced Cry1B.868 protein sample using LC-MS/MS. In total, 96% coverage (1156 out of 1199 amino acids) of the expected protein sequence was covered by the identified peptides. Gray highlighted regions correspond to the receptor binding domain see Appendix L.

C.1.4. Results of Western Blot Analysis of the Cry1B.868 Protein Isolated from the Grain of MON 95379 and Immunoreactivity Comparison to *Bt*-produced Cry1B.868 Protein

Western blot analysis was conducted using rabbit anti-Cry1B.868 polyclonal antibody as additional means to confirm the identity of the Cry1B.868 protein isolated from the grain of MON 95379 and to assess the equivalence of the immunoreactivity of the MON 95379-produced and *Bt*-produced Cry1B.868 proteins.

The results showed that immunoreactive bands with the same electrophoretic mobility were present in all lanes loaded with the MON 95379-produced and *Bt*-produced Cry1B.868 proteins (Figure C-3). For each amount loaded, comparable signal intensity was observed between the MON 95379-produced and *Bt*-produced Cry1B.868 protein bands. As expected, the signal intensity increased with the increasing amounts of protein loaded, thus, supporting identification of MON 95379-produced Cry1B.868 protein.

To compare the immunoreactivity of the MON 95379-produced and *Bt*-produced Cry1B.868 proteins, densitometric analysis was conducted on the bands that migrated at the expected apparent MW for Cry1B.868 proteins (~ 130 kDa). The signal intensity (reported in OD) of the band of interest in lanes loaded with MON 95379-produced and *Bt*-produced Cry1B.868 proteins was measured. Because the mean signal intensity of the MON 95379-produced Cry1B.868 protein was within 35% of the mean signal intensity of the *Bt*-produced Cry1B.868 protein (Table C-4), the MON 95379-produced Cry1B.868 and *Bt*-produced Cry1B.868 proteins were determined to have equivalent immunoreactivity.

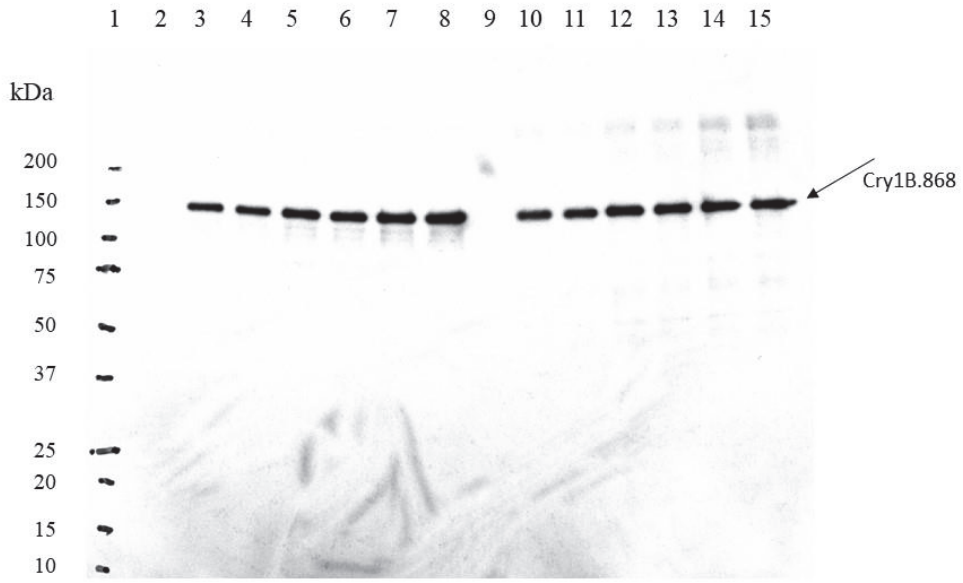

Figure C-3. Western Blot Analysis and Immunoreactivity of MON 95379-Produced and *Bt*-Produced Cry1B.868 Proteins

Aliquots of the MON 95379-produced and *Bt*-produced Cry1B.868 proteins were subjected to SDS-PAGE and electro-transferred to a PVDF membrane. Proteins were detected using rabbit anti-Cry1B.868 polyclonal antibodies as the primary antibodies. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system. The 1-minute exposure is shown. The approximate MW (kDa) of the standards are shown on the left. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Blank	-
3	MON 95379-produced Cry1B.868	5
4	MON 95379-produced Cry1B.868	5
5	MON 95379-produced Cry1B.868	10
6	MON 95379-produced Cry1B.868	10
7	MON 95379-produced Cry1B.868	15
8	MON 95379-produced Cry1B.868	15
9	Blank	-
10	*Bt*-produced Cry1B.868	5
11	*Bt*-produced Cry1B.868	5
12	*Bt*-produced Cry1B.868	10
13	*Bt*-produced Cry1B.868	10
14	*Bt*-produced Cry1B.868	15
15	*Bt*-produced Cry1B.868	15

Table C-4. Immunoreactivity of the MON 95379-Produced and *Bt*-Produced Cry1B.868 Proteins

Mean Signal Intensity from MON 95379-Produced Cry1B.868 [1] (OD)	Mean Signal Intensity from *Bt*-Produced Cry1B.868 [1] (OD)	Acceptance Limits[2] (OD)
2156.21	2527.92	1643.15 – 3412.69

[1] Each value represents the mean of six values (n = 6).
[2] The acceptance limits are for the MON 95379-produced Cry1B.868 protein and are based on the interval between -35% (2527.92 x 0.65 = 1643.15) and +35 % (2527.92 x 1.35 = 3412.69) of the mean of the *Bt*-produced Cry1B.868 signal intensity across all loads.

C.1.5. Results of the Cry1B.868 Protein Molecular Weights and Purity Analysis

For apparent MW and purity determination, the MON 95379-produced Cry1B.868 and the *Bt*-produced Cry1B.868 proteins were subjected to SDS-PAGE. Following electrophoresis, the gel was stained with Brilliant Blue G-Colloidal stain and analyzed by densitometry. The MON 95379-produced Cry1B.868 protein (Figure C-4, lanes 3-8) migrated to the same position on the gel as the *Bt*-produced Cry1B.868 protein (Figure C-4, lane 2) and the apparent MW was calculated to be 126.8 kDa (Table C-5). Because the experimentally determined apparent MW of the MON 95379-produced Cry1B.868 protein was within the acceptance limits for equivalence (Table C-6), the MON 95379-produced Cry1B.868 and *Bt*-produced Cry1B.868 proteins were determined to have equivalent apparent molecular weights.

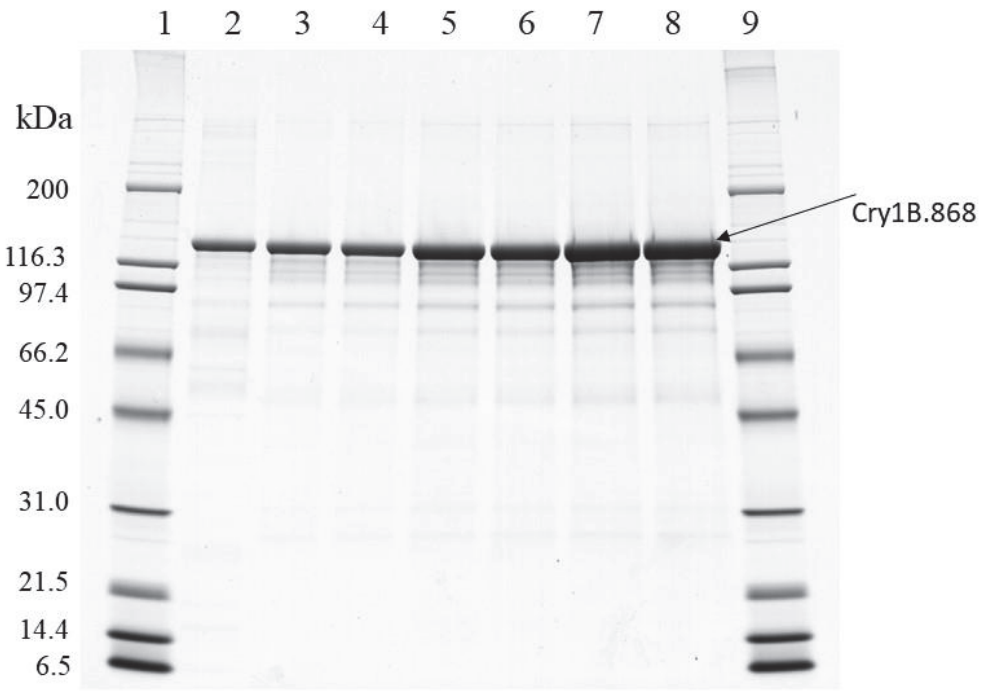

Figure C-4. Purity and Apparent Molecular Weight Analysis of the MON 95379-Produced Cry1B.868 Proteins

Aliquots of the MON 95379-produced and the *Bt*-produced Cry1B.868 proteins were subjected to SDS-PAGE and the gel was stained with Brilliant Blue G-Colloidal stain. The MWs (kDa) are shown on the left and correspond to the standards loaded in lanes 1 and 9. Lane 10 was cropped from the image. The intact Cry1B.868 protein is indicated with an arrow in the image. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standard	5.0
2	*Bt*-produced Cry1B.868	1.0
3	MON 95379-produced Cry1B.868	1.0
4	MON 95379-produced Cry1B.868	1.0
5	MON 95379-produced Cry1B.868	2.0
6	MON 95379-produced Cry1B.868	2.0
7	MON 95379-produced Cry1B.868	3.0
8	MON 95379-produced Cry1B.868	3.0
9	Broad Range MW Standard	5.0
10	Blank	

Table C-5. Apparent Molecular Weight and Purity Analysis of the MON 95379-Produced Cry1B.868 Protein

	Apparent MW[1] (kDa)	Purity[2] (%)
Average (n = 6)	126.8	97

[1]Final MW was rounded to one decimal place.
[2]Average % purity was rounded to the nearest whole number.

Table C-6. Apparent Molecular Weight Comparison Between the MON 95379-Produced Cry1B.868 and *Bt*-Produced Cry1B.868 Proteins

Apparent MW of MON 95379-Produced Cry1B.868 Protein (kDa)	Apparent MW of *Bt*-Produced Cry1B.868 Protein[1] (kDa)	Acceptance Limits[2] (kDa)
126.8	129.6	118.5 – 140.7

[1] As reported on the COA of the *Bt*-produced Cry1B.868 protein (lot 7565).
[2] Data obtained for the *Bt*-produced Cry1B.868 protein were used to generate the prediction interval. Values in this column represent a 95% prediction interval developed from eight individual assays with *Bt*-produced Cry1B.868 protein (Section C.2.3.6. and Table C-8).

C.1.6. Cry1B.868 Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether the Cry1B.868 protein was glycosylated when expressed in the maize grain of MON 95379, the MON 95379-produced Cry1B.868 protein was analyzed using an ECL™ glycoprotein detection method. Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 95379-produced and *Bt*-produced Cry1B.868 proteins, the *Bt*-produced Cry1B.868 protein was also analyzed.

A clear glycosylation signal was observed at the expected molecular weight (~ 80 kDa) in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure C-5, Panel A). In contrast, no glycosylation signal was observed in the lanes containing the *Bt*-produced Cry1B.868 protein or MON 95379-produced Cry1B.868 protein (Figure C-5, Panel A).

To confirm that MON 95379-produced Cry1B.868 and *Bt*-produced Cry1B.868 proteins were appropriately loaded for glycosylation analysis, a second membrane with identical loadings and transfer time was stained with Coomassie Blue R250 for protein detection. Both the MON 95379-produced and *Bt*-produced Cry1B.868 proteins were detected (Figure C-5, Panel B). These data indicate that the glycosylation status of MON 95379-produced Cry1B.868 protein is equivalent to that of the *Bt*-produced Cry1B.868 protein and that neither is glycosylated.

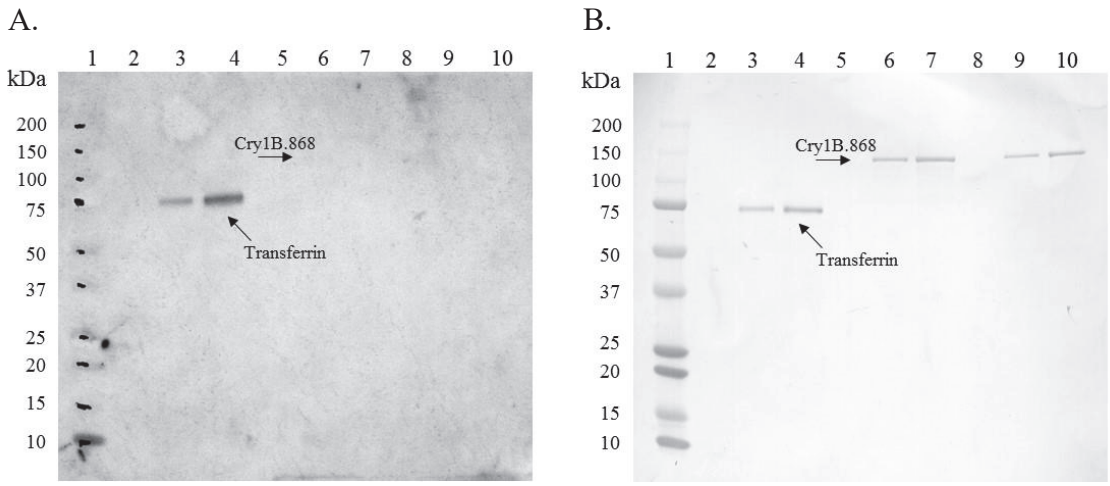

Figure C-5. Glycosylation Analysis of the MON 95379-Produced and *Bt*-Produced Cry1B.868 Proteins

Aliquots of the transferrin (positive control), MON 95379-produced and *Bt*-produced Cry1B.868 proteins were subjected to SDS-PAGE and electro-transferred to a PVDF membrane. The MWs (kDa) correspond to the Precision Plus Protein™ Standards. The arrows show the expected migration of the MON 95379-produced and *Bt*-produced Cry1B.868 proteins and transferrin. (A) Where present, the labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based detection using ECL reagents and exposure to Hyperfilm™. The 1.5-minute exposure is shown. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Blank	-
3	Transferrin (positive control)	100
4	Transferrin (positive control)	200
5	Blank	-
6	MON 95379-produced Cry1B.868	100
7	MON 95379-produced Cry1B.868	200
8	Blank	-
9	*Bt*-produced Cry1B.868	100
10	*Bt*-produced Cry1B.868	200

C.1.7. Cry1B.868 Functional Activity

The MON 95379-produced Cry1B.868 and *Bt*-produced Cry1B.868 proteins were considered to have equivalent functional activity if the activities of both proteins (measured by EC_{50} values) were within the acceptance limits of 0.024 to 0.272 µg of Cry1B.868 protein/mL diet, which is the prediction interval calculated from a data set of *Bt*-produced Cry1B.868 protein activity.

The experimentally determined functional activity for the MON 95379-produced and *Bt*-produced Cry1B.868 proteins are presented in Table C-7. The mean EC_{50}s values of MON 95379-produced and *Bt*-produced Cry1B.868 proteins were 0.15 and 0.20 µg of Cry1B.868 protein/mL diet, respectively. Because both EC_{50}s fall within the preset acceptance limits (Table C-7), the MON 95379-produced Cry1B.868 protein was considered to have equivalent functional activity to that of the *Bt*-produced Cry1B.868 protein.

Table C-7. Functional Activity of MON 95379-Produced and *Bt*-Produced Cry1B.868 Proteins

Replicates	MON 95379-Produced Cry1B.868 EC_{50} [1] (µg protein/ml diet)	*Bt*-Produced Cry1B.868 EC_{50} [1] (µg protein/ml diet)	EC_{50} Acceptance Limits[2] (µg protein/ml diet)
1	0.18	0.25	
2	0.15	0.21	0.024 – 0.272
3	0.12	0.14	
Mean	0.15	0.20	-

[1] Value refers to mean EC_{50} calculated based on three independent assays (n = 3).
[2] Data obtained for the *Bt*-produced Cry1B.868 proteins were used to generate a prediction interval for setting the acceptance limits. Values in this column represent a 95% prediction interval developed from 24 individual bioassays with *Bt*-produced Cry1B.868 protein (Section C.2.3.6. and Table C-9).

C.1.8. Cry1B.868 Protein Identity and Equivalence Conclusion

The MON 95379-produced Cry1B.868 protein purified from MON 95379 grain was characterized, and a comparison of the physicochemical and functional properties between the MON 95379-produced and the *Bt*-produced Cry1B.868 proteins was conducted following a panel of analytical tests: 1) N-terminal sequence analysis established the same identity for the MON 95379-produced and the *Bt*-produced Cry1B.868 proteins; 2) Nano LC-MS/MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the *cry1B.868* gene product present in MON 95379; 3) the MON 95379-produced and the *Bt*-produced Cry1B.868 proteins were both detected on a western blot probed with antibodies specific for Cry1B.868 protein and the immunoreactive properties of both proteins was shown to

be equivalent; 4) the electrophoretic mobility and apparent molecular weight of the MON 95379-produced and the *Bt*-produced Cry1B.868 proteins were shown to be equivalent; 5) the glycosylation status of MON 95379-produced and the *Bt*-produced Cry1B.868 proteins was determined to be equivalent; and 6) the functional activity of the MON 95379-produced and the *Bt*-produced Cry1B.868 was demonstrated to be equivalent. These results demonstrate that the MON 95379-produced and the *Bt*-produced Cry1B.868 protein are equivalent.

The ability of the Cry1B.868 to bind to its target receptor is dependent on the confirmation of the Cry1B.868 receptor binding domain. Protein structure, or confirmation, is determined by the specific sequence of amino acids that comprise the different structural and functional elements. It can inferred that once properly folded, proteins that possess equivalent amino acid sequences would have equivalent functional activity or receptor binding. The MON 95379- and *Bt*-produced Cry1B.868 proteins possess equivalent amino sequences, and thereby equivalent receptor binding domains, as evidenced by the very high amino acid sequence similarity seen by mass fingerprint analysis where >90% coverage was observed. The equivalency of the protein sequences was also confirmed indirectly through sequencing the of genes of the expressed MON 95379- and *Bt*-produced Cry1B.868 proteins. Finally, the equivalent functional activity of the protein isolated from both sources indicates that the confirmation of the receptor binding domains in both proteins are equivalent.

Taken together, the equivalency data provided in support of MON 95379 Cry1B.868 confirms that the *Bt*-produced Cry1B.868 protein is appropriate surrogate for use in the evaluation of the safety of the MON 95379-produced Cry1B.868 protein.

C.2. Materials and Methods for Characterization of Cry1B.868 Protein Produced in MON 95379

C.2.1. Materials

The test substance was the MON 95379-produced Cry1B.868 protein (lot 8210) purified from grain of MON 95379 (lot 11479279). The grain was produced by Bayer Company. The MON 95379-produced Cry1B.868 protein was stored in a -80 ºC freezer in a buffer containing 20 mM CAPS, pH 11.5, 10 mM DTT, 240 mM NaCl, and 1 mM Benzamidine.

The reference substance was the *Bt*-produced Cry1B.868 (lot 7565, historical Orion lot 11479349). The Cry1B.868 reference substance was generated from the cell paste produced by fermentation of *Bt* containing the pMON236906 expression plasmid. The DNA sequence encoding Cry1B.868 protein that is contained on the expression plasmid was confirmed both prior to and following fermentation.

C.2.2. Protein Purification

The plant-produced Cry1B.868 protein used in this equivalence study was purified from the grain of MON 95379 maize. The purification procedure was not performed under a GLP plan; however, all procedures were documented in ELN and, where applicable, SOPs were followed. Because of

the physicochemical similarity between Cry1B.868 and Cry1Da_7 proteins, a purification process was developed to co-isolate these two proteins in the front purification steps, i.e., extraction from the maize flour followed by Q Sepharose Fast Flow (QSFF; GE Healthcare, Piscataway, NJ) chromatography. The resulting fractions from the QSFF chromatography were applied to immuno-affinity chromatography (IAC) to purify Cry1B.868 and Cry1Da_7 separately with their respective antibodies. A detailed description of the purification procedure specific for Cry1B.868 protein was archived under lot 8210 in the Bayer Regulatory Archives and a brief purification procedure was described below. All purification steps were performed at ~4 °C, except where specifically stated.

Grain of MON 95379 was ground to fine powder in the presence of dry ice and stored at -80 °C until use. A total of ~30 kg of ground powder was processed in three different runs. In each run, ~10 kg of ground powder was mixed with 100 L of extraction buffer (50 mM Na_2CO_3, pH ~11.3, 5 mM DTT, 2 mM EDTA, 2 mM Benzamine-HCl, 0.5 mM AEBSF, and 0.15 M NaCl) for ~2 hr. The slurry was clarified by centrifugation at 13,500 × g for 1 h. The supernatant (~90 L) was loaded onto a ~3 L QSFF column that was equilibrated with buffer A (50 mM Carbonate/Bicarbonate, pH 9.0, 5 mM DTT, 2 mM EDTA, 2 mM benzamidine-HCl, and 0.1 mM AEBSF). After a washing step with 0.1 M NaCl, bound proteins were eluted with a linear salt gradient from 0.1 M to 1.0 M NaCl over a volume of ~30 L. The fraction pool (~7 L) that contained both Cry1B.868 and Cry1Da_7 was then concentrated and buffer-exchanged to the IAC binding buffer (50 mM Carbonate/Bicarbonate, pH 9.0, 2 mM EDTA, 2 mM benzamidine-HCl, and 0.15 M NaCl,), using a 30,000 NMWC Hollow Fiber Cartridge (GE Healthcare). The end volume was ~1L.

To purify Cry1B.868 and Cry1Da_7 from the buffer-exchanged QSFF fraction pool (~1 L), the pool was first loaded onto a Cry1Da_7 immuno-affinity column. The Cry1Da_7 protein bound on the column was recovered through a separate procedure. The flow-through with un-bound Cry1B.868 was collected and loaded onto a Cry1B.868 immuno-affinity column of ~50 ml MabSelect Protein A resin (GE Healthcare, Cat #: 17-5199-02) cross-linked with ~100 mg of anti-Cry1B.868 monoclonal antibody. After washing with a buffer containing 50 mM Tris, pH 8, 0.75 M $(NH_4)_2SO_4$, 2 mM EDTA and 2 mM benzamidine, Cry1B.868 was then eluted with polyol buffer [50 mM Tris, pH 8.0, 0.75 M $(NH_4)_2SO_4$, 40% propylene glycol, 2 mM EDTA, and 2 mM benzamidine]. Fractions containing Cry1B.868 protein as identified by SDS-PAGE and western blot were pooled and stored in -80°C until further process.

The purified Cry1B.868 protein samples from the three runs were separately concentrated and buffer exchanged to storage buffer (20 mM CAPS, pH 11.5, 10 mM DTT, 240 mM NaCl, and 1 mM Benzamidine) using 15 ml Amicon Ultracel-30K centrifugal concentration device (Millipore, Tullagreen, Ireland). The resulting samples were then combined as the final Cry1B.868 protein product. This Cry1B.868 protein, purified from the grain of MON 95379, was aliquoted, assigned lot 8210, and stored in a -80 °C freezer.

C.2.3. Methods for Characterization

C.2.3.1. N-Terminal Sequencing/Mass Fingerprint Analysis

The test substance was analyzed for N-terminal amino acid sequence and peptide mass fingerprint by LC-MS/MS. Peptide fragments produced by digestion with trypsin or Asp-N protease were analyzed. An aliquot of the test substance was subjected to SDS-PAGE and Cry1B.868 was visualized on the gel with InstantBlue™ (Expedeon, Cambridgeshire, UK) staining followed by de-staining with Milli-Q water. Gel bands containing Cry1B.868 protein (~4 µg in each gel band) were excised, de-stained, incubated in ~200 µl of 100 mM ammonium bicarbonate for 30 min at room temperature (RT) for each gel band. The protein in each gel band was reduced by incubation in 100 µl of 10 mM DTT at ~37°C for 1 hr, alkylated by incubation in the dark for 30 min at RT with 100 µl of 20 mM iodoacetamide, and then incubated in 200 µl of 25 mM ammonium bicarbonate for ~15 min with agitation at RT for three times. Each gel band was dried down, rehydrated in 20 µl of either 20 µg/ml of trypsin or 20 µg/ml of Aps-N protease solution and incubated at RT for 1 hr to allow the solution to diffuse into the gel band. After incubation, removed excess liquid; 40 µl of 25 mM ammonium bicarbonate/10% acetonitrile solution was added to each gel band and the reaction was incubated at 37°C for ~17 hr. After incubation, 1 µl of formic acid was added to stop the digestion; each tube was sonicated for 6 min and the supernatant was collected and transferred to a microcentrifuge tube. The digested gel band was extracted with 30 µl of 70% acetonitrile/0.1% formic acid (FA) twice. The samples were completely dried using vacuum centrifugation and then solubilized in 10 µl of 0.1 % FA for LC-MS/MS analysis.

The LC-MS/MS data acquisition was performed on a Dionex 3000 Ultimate nano LC- system (Dionex) interfaced to an orbitrap fusion mass spectrometer (Thermo Scientific), which is equipped with a nano-ESI (electrospray ionization) source. The sample was loaded and separated online using an Acclaim PepMap100 C18 nano column (75 µm id × 150 mm, 2 µm, 100 Å, Dionex). The separation of the digests was achieved at a 300 nl/min flow rate using an acetonitrile gradient as follows: 0-4 min sustaining 2% solvent B, 4-80 min ramping solvent B 2-40%, 80-85 min ramping solvent B 40-90%. The column was washed/equilibrated between injections as follows: 85-90 min maintaining solvent B at 90%, 90-90.1 min decreasing solvent B 90-2%, and 90.1-100 min maintaining solvent B at 2%. Solvent A was 0.1% FA in water. Solvent B was 0.1% FA in acetonitrile.

The orbitrap fusion mass spectrometer was operated with data dependent acquisition method. The first scan event was a full Fourier transform mass spectrometry scan with a mass resolution of 120,000 at m/z of 200. The second scan event was a tandem mass spectrometry scan of fragments from collision induced dissociation (CID) of precursor ions from the first scan event with an isolation width of 2.0 m/z.

The LC-MS/MS dataset were analyzed using Mascot (version 2.5, Matrix Science). MS/MS spectra were searched against the theoretical Cry1B.868 protein sequence. The mass tolerances of MS1 mass and MS/MS mass were set as 5 ppm and 0.5 Da, respectively. Carboxymethylation of cysteine was set as a fixed modification. Methionine oxidation and N-terminal amino acid

acetylation were set as variable modifications. A maximum of two missed cleavages for the protease used were allowed. The mass coverage was accepted if it is greater than 40% of the protein sequence.

C.2.3.2. Western Blot Analysis-Immunoreactivity

Western blot analysis was performed as follows to confirm the identity of the MON 95379-produced Cry1B.868 protein and to compare the immunoreactivity of the MON 95379-produced and *Bt*-produced Cry1B.868 protein. MON 95379-produced and *Bt*-produced Cry1B.868 proteins were diluted to 1 ng/µl (purity-corrected intact Cry1B.868 protein concentration) in 5 × LB and water to obtain a final concentration of 1 × LB and heated to 95-105 °C for 5 min. Three amounts (~5, ~10, and ~15 ng) of the intact test substance (total protein concentration × purity of the intact Cry1B.868 protein) and the intact reference substance (total protein concentration × purity of the intact Cry1B.868 protein) were loaded in duplicate onto a pre-cast Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight standards (Precision Plus Protein Standards™, Bio-Rad) were loaded on the gel for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

The western blotting procedure was performed using an iBind™ Western System apparatus (Life Technologies, Grand Island, NY). The membrane was blocked with 1 × iBind™ Solution (Life Technologies) and incubated with rabbit anti-Cry1B.868 polyclonal antibody (lot 29121) at a dilution of 1:1000 in 1 × iBind™ Solution. After washing with 1 × iBind™ Solution, the membrane was next incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:1000 in 1 × iBind™ Solution and washed again with 1 × iBind™ Solution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare) and exposed to Hyperfilm™ ECL high performance chemiluminescence film (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica, Tokyo, Japan).

Quantification of the bands on the film was performed on a Bio-Rad GS-900 densitometer with the supplied Image Lab 5.0 Security Edition software using the volume tool. The signal intensity of the immunoreactive bands migrating at the expected position for the intact Cry1B.868 protein was quantified as adjusted volume values. The immunoreactivity was reported in OD, which equals to the total sum of the intensity of the pixels inside the volume boundary.

C.2.3.3. Apparent Molecular Weight and Purity Determination by SDS-PAGE

MON 95379-produced and *Bt*-produced Cry1B.868 proteins were diluted in 5 × loading buffer [LB, 0.31 M Tris-Cl, pH 7.5, 10% SDS, 50% glycerol, 25% (v/v) 2-mercaptoethanol, 0.025% (w/v) Bromophenol blue] and Milli-Q water and heated to 95-105°C for 5 min. The MON 95379-produced Cry1B.868 protein was loaded in duplicate at ~1.0, ~2.0, and ~3.0 µg based on total protein concentration, onto a Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen, Carlsbad, CA) in lanes 3, 4, 5, 6, 7 and 8. The *Bt*-produced Cry1B.868 protein was loaded at

~1.0 µg total protein in lane 2. Broad Range Molecular Weight Standards (Bio-Rad) were prepared and loaded in lanes 1 and 9 on the gel. Following electrophoresis at a constant voltage, proteins were briefly fixed in 40% (v/v) methanol, 7% (v/v) acetic acid and stained for 18 ± 2 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO). Gels were briefly destained in 10% (v/v) acetic acid, 25% (v/v) methanol followed by 8 ± 2 hr in 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-900 densitometer supplied with Image Lab 5.0 Security Edition software. The molecular weight of the test substance was calculated based on the mean of the molecular weight of the major band of Cry1B.868 (~130 kDa) in all six lanes containing the test substance. The purity of the test substance was calculated based on multiple stained bands corresponding to the Cry1B.868 protein including a major band (~130 kDa) and three minor bands (~120, ~110, and ~100 kDa) recognized by western blot analysis. The purity was reported as an average of all 6 lanes containing the MON 95379-produced Cry1B.868 protein.

C.2.3.4. Glycosylation Analysis

An ECL glycoprotein detection method (GE Healthcare) was used for glycoprotein detection. The MON 95379-produced Cry1B.868 protein, the *Bt*-produced Cry1B.868 protein and a positive control, transferrin (Sigma) were diluted in 1 × loading buffer and then heated to 95 -105 °C for 5 min. Two amounts (~ 100 ng and ~ 200 ng) of the intact MON 95379-produced Cry1B.868 protein (purity-corrected), the intact *Bt*-produced Cry1B.868 protein (purity-corrected), and the positive control were loaded onto a pre-cast Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight markers (Precision Plus Protein™ Standards, Bio-Rad) were also loaded for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

Glycosylation analysis was performed on the PVDF membrane at room temperature using the ECL Glycoprotein Detection Module (GE Healthcare) as directed by the manufacturer. The labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based detection using ECL reagents (GE Healthcare) and exposure to Hyperfilm™ (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta). An identical gel was run and electrotransferred to a PVDF membrane in parallel. Proteins were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) and then destained with equivalent chemical reagents to 1× Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad). After washing with water, the blot was scanned using a Bio-Rad GS-900 densitometer with the supplied Image Lab 5.0 Security Edition Software.

C.2.3.5. Functional Activity

Insects. Fall armyworm (FAW, Spodoptera frugiperda) is an appropriate test system due to its susceptibility to the Cry1B.868 protein. FAW were obtained from Bayer (Union City, TN). Insect eggs were incubated at temperatures ranging from 10°C to 27°C, to achieve the desired hatch time.

Bioassays. FAW larvae were used to measure the functional activity of the MON 95379-produced Cry1B.868 and the *Bt*-produced Cry1B.868 in accordance with ME-0044-04. The MON 95379-

produced Cry1B.868 and the *Bt*-produced Cry1B.868 substances were analyzed concurrently in each bioassay replicate. Bioassay replicates were initiated on different days, each using a separate batch of insects. Each bioassay replicate consisted of a series of six dilutions yielding a dose series with a two-fold separation factor ranging from 0.012 – 0.4 µg Cry1B.868/ml diet for the *Bt*-produced Cry1B.868 and 0.014 – 0.47 Cry1B.868/ml diet for the MON 95379-produced Cry1B.868 and an assay (buffer) control. Dosing solutions (5.0 ml) were prepared by diluting the protein sample with purified water and incorporating the dilution into an agar-based FAW diet (Southland, Village Lake, AR) to a final volume of 25 ml. Each diet mixture was dispensed in 1 mL aliquots into 16 wells of a 128-well tray (Frontier Agricultural Services). Each well was targeted to be infested with a single FAW larva (≤ 30 hours after first observation of hatching). Larvae were allowed to feed for a period of seven days in an environmental chamber programmed at 27ºC, 60% relative humidity, and a lighting regime of 14 hours light: 10 hours dark. The number of FAW larvae infested, the number of surviving larvae, and the combined mass of the surviving larvae were recorded for each treatment at the end of the seven-day incubation period.

The functional activity, measured as an EC_{50}, of the test substance and reference substance was characterized using a fall armyworm insect bioassay. The EC_{50} value is defined as the concentration that results in 50% growth The EC_{50} value is defined as the concentration that results in 50% growth inhibition. Logistic regression was used to estimate the EC_{50} values for growth inhibition for each bioassay. The insect bioassay was considered acceptable if less than or equal to 20% mortality was observed for the vehicle control and if >50% growth inhibition was observed in the highest tested protein concentration level.

C.2.3.6. Prediction Intervals as Acceptance Criteria

Acceptance criteria (acceptance limits) based on prediction intervals were used to assess the equivalence of the MON 95379-produced and *Bt*-produced Cry1B.868 proteins for apparent MW and functional activity. A prediction interval is an estimate of an interval in which a randomly selected future observation from a population will fall, with a certain degree of confidence, given what has already been observed; *i.e.*, prediction intervals are generated based on statistical analysis of existing data (Urquhart et al., 2015).

The source of the data used to generate the prediction intervals for apparent MW and functional activity for Cry1B.868 protein equivalence assessment are provided in Table C-8 and Table C-9, respectively. The data used were generated under GLP guidelines and included apparent MW and functional activity estimates for the reference *Bt*-produced Cry1B.868 protein.

The two-sided 95% prediction interval (PI) for one future assay was calculated using SAS software or an ELN template validated against SAS (2012, Software Release 9.4 (TS1M4), SAS Institute) according to the guidance document of Ramírez (2009).

Table C-8. Individual Assay Data and 95% Prediction Interval Generated for the Apparent MW of Cry1B.868 Protein for One Future Assay

Assay Number[1]	Apparent MW of Cry1B.868 Protein (kDa)[2]

1	130.9
2	134.4
3	133.7
4	133.4
5	124.6
6	124.8
7	124.1
8	131.1
Mean	129.6
Standard Deviation	4.4
95% Prediction Interval	**118.5 – 140.7**
99% Prediction Interval	113.2 – 146.0

[1] Assay 1-8 represents the MW data from the initial characterization of *Bt*-produced Cry1B.868 (lot 7565).
[2] The values in the table represent the mean of six (n = 6) data points within each assay. With 95% or 99% confidence, the mean of the 6 data points from the next single assay of the population will fall within the stated interval. Mean and prediction interval values rounded to one decimal point.

Table C-9. Estimated EC_{50} Values from 24 Dose-Response Diet-Incorporation Insect Bioassays of the Cry1B.868 Protein

Assay Number[1]	EC_{50} (µg protein/ml diet)
1	0.095
2	0.067
3	0.129
4	0.050
5	0.072
6	0.040
7	0.062
8	0.091
9	0.042
10	0.054
11	0.089
12	0.172
13	0.083
14	0.103
15	0.070
16	0.186
17	0.051
18	0.038
19	0.084
20	0.097
21	0.138
22	0.062
23	0.091
24	0.187
Mean	0.081
95% Prediction Interval	**0.024 – 0.272**
99% Prediction Interval	0.017 – 0.379

[1] Assay 1-24 represents functional activity data from the characterization of *Bt*-produced Cry1B.868 (lot 7565).

References for Appendix C

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Appendix D: Characterization of Cry1Da_7 Protein Produced in MON 95379

D.1. Characterization of the Cry1Da_7 Protein

D.1.1. Cry1Da_7 Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties and confirmation of the safety of the introduced protein(s). For the safety data generated using the *Bt*-produced Cry1Da_7 protein to be applied to the MON 95379-produced Cry1Da_7 protein (plant-produced Cry1Da_7), the equivalence of the plant- and *Bt*-produced proteins must first be demonstrated. To assess the equivalence between the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins, a small quantity of the MON 95379-produced Cry1Da_7 protein was purified from MON 95379 grain. The MON 95379-produced Cry1Da_7 protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins was assessed using a panel of analytical tests; as shown in Table D-1. Taken together, these data provide a detailed characterization of the MON 95379-produced Cry1Da_7 protein and establish the equivalence of the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins. Based on this established equivalence, conclusions derived from digestibility, heat susceptibility and oral acute toxicology, and non-target organism studies conducted with *Bt*-produced Cry1Da_7 protein are applicable to MON 95379-produced Cry1Da_7 protein.

Table D-1. Summary of MON 95379 Cry1Da_7 Protein Identity and Equivalence

Analytical Test	Assessment	Analytical Test Outcome
N-terminal sequence	Identity	The expected N-terminal sequence for MON 95379-produced Cry1Da_7 protein was observed by Nano LC-MS/MS[1]
Nano LC-MS/MS[1]	Identity	Nano LC-MS/MS[1] analysis of trypsin digested peptides from MON 95379-produced Cry1Da_7 protein yielded peptide masses consistent with expected peptide masses from the theoretical trypsin digest of the amino acid sequence
Western blot analysis	Identity and Equivalence	MON 95379-produced Cry1Da_7 protein identity was confirmed using a western blot probed with antibodies specific for Cry1Da_7 protein Immunoreactive properties of the MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7 proteins were shown to be equivalent
Apparent molecular weight (MW)	Equivalence	Electrophoretic mobility and apparent molecular weight of the MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7 proteins were shown to be equivalent
Glycosylation analysis	Equivalence	Glycosylation status of MON 95379-produced Cry1Da_7 and *Bt*-produced Cry1Da_7 proteins were shown to be equivalent
Functional activity	Equivalence	Functional activity of the MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7 proteins were shown to be equivalent by insect bioassay

[1] Nano LC-MS/MS = Nanoscale liquid chromatography-tandem mass spectrometry

The details of the materials and methods for the panel of analytical tests used to evaluate and compare the properties of the MON 95379-produced Cry1Da_7 and *Bt*-produced Cry1Da_7 proteins are described at the end of Appendix D. A sumary of the data obtained to support a conclusion of protein equivalence is provided below.

D.1.2. Results of the N-Terminal Sequencing Analysis

The expected N-terminal sequence for the Cry1Da_7 protein deduced from the *cry1Da_7* gene present in maize of MON 95379 was observed by LC-MS/MS, except that the N-terminal methionine was cleaved in vivo from MON 95379 produced Cry1Da_7 by methionine aminopeptidase or other aminopeptidases (see Experimental Sequence, Figure D-1). The cleavage of the N-terminal methionine from proteins in vivo by methionine aminopeptidase is common in many organisms (Bradshaw et al., 1998; Wang et al., 2016). The N-terminal sequence for MON 95379-produced Cry1Da_7 protein was consistent with the N-terminal sequence for the *Bt*-produced Cry1Da_7 protein observed by LC-MS/MS (Figure D-1). Hence, the sequence information confirms the identity of the Cry1Da_7 protein isolated from the grain of MON 95379.

Amino Acids Residue # from the N-terminus	→	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Bt-produced Cry1Da_7 sequence	→	-	A	E	I	N	N	Q	N	Q	C	V	P	Y	N	C	L
Expected Cry1Da_7 Sequence	→	M	A	E	I	N	N	Q	N	Q	C	V	P	Y	N	C	L
MON 95379 Experimental Sequence	→	-	A	E	I	N	N	Q	N	Q	C	V	P	Y	N	C	L

Figure D-1. N-Terminal Sequence of the MON 95379-Produced Cry1Da_7 Protein

The experimental sequence obtained from the MON 95379-produced Cry1Da_7 was compared to the expected sequence deduced from the *cry1Da_7* gene present in MON 95379. *Bt*-produced Cry1Da_7 protein sequence above was derived from the reference substance COA (lot 7799). The single letter International Union of Pure and Applied Chemistry - International Union of Biochemistry (IUPAC-IUB) amino acid code is M, Methionine; A, Alanine; E, Glutamic acid; I, Isoleucine; N, Asparagine; Q, Glutamine; C, Cysteine; V, Valine; P, Proline; Y, Tyrosine; L, Leucine.

D.1.3. Results of Mass Fingerprint Analysis

Peptide mass fingerprint analysis is a standard technique used for confirming the identity of proteins. The identity of the MON 95379-produced Cry1Da_7 protein was confirmed by LC-MS/MS analysis of peptide fragments produced by the trypsin digestion of the MON 95379-produced Cry1Da_7 protein.

There were 113 unique peptides identified that corresponded to the masses expected to be produced by trypsin digestion of the MON 95379-produced Cry1Da_7 protein (Table D-2, Panel A). The identified masses were used to assemble a coverage map of the entire Cry1Da_7 protein (Figure D-2, Panel A). The experimentally determined coverage of the MON 95379-produced Cry1Da_7 protein was 89% (Figure D-2, Panel A, 1049 out of 1166 amino acids). This analysis further confirms the identity of MON 95379-produced Cry1Da_7 protein.

There were 136 unique peptides identified that corresponded to the masses expected to be produced by trypsin digestion of the *Bt*-produced Cry1Da_7 protein (Table D-3) by LC-MS/MS analysis during the protein characterization. The identified masses were used to assemble a coverage map of the entire Cry1Da_7 protein (Figure D-2, Panel B). The experimentally determined coverage of the *Bt*-produced Cry1Da_7 protein was 89% (Figure D-2, Panel B, 1049 out of 1166 amino acids). This analysis further confirms the identity of *Bt*-produced Cry1Da_7 protein.

Table D-2. Summary of the Tryptic Masses Identified for the MON 95379-Produced Cry1Da_7 Using LC-MS/MS[1]

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
2304.0267	2304.0263	0.0004	2 - 20	AEIN...SNPK
3243.5301	3243.5288	0.0013	2 - 28	AEIN...GEER
957.5145	957.5131	0.0014	21 - 28	EIILGEER
763.3866	763.3864	0.0002	88 - 93	IEEFAR
687.3664	687.3664	0.0000	94 - 99	NQAISR
1035.5602	1035.5600	0.0002	100 - 108	LEGL...NLYK
535.3120	535.3118	0.0002	109 - 112	VYVR
1745.8390	1745.8373	0.0017	113 - 127	AFSD...PALR
2291.0670	2291.0640	0.0030	113 - 131	AFSD...EEMR
882.4554	882.4559	-0.0005	120 - 127	DPTNPALR
1427.6810	1427.6827	-0.0017	120 - 131	DPTN...EEMR
2063.0863	2063.0874	-0.0011	132 - 149	IQFN...PLFR
2712.5042	2712.4962	0.0080	150 - 173	VQNY...SILR
3601.9240	3601.9256	-0.0016	150 - 181	VQNY...FGER
907.4401	907.4400	0.0001	174 - 181	DVSVFGER
1309.6070	1309.6051	0.0019	182 - 192	WGYD...INNR
2868.3482	2868.3501	-0.0019	193 - 216	YSDL...QGLR
3024.4491	3024.4512	-0.0021	193 - 217	YSDL...GLRR
629.3609	629.3609	0.0000	217 - 221	RLEGR
1311.6614	1311.6612	0.0002	222 - 231	FLSD...VYNR
1614.8294	1614.8307	-0.0013	222 - 233	FLSD...NRFR
2379.2982	2379.2951	0.0031	234 - 253	RQLT...YDIR
2223.1952	2223.1940	0.0012	235 - 253	QLTI...YDIR
1391.7412	1391.7409	0.0003	254 - 265	TYPI...QLTR
3409.7462	3409.7445	0.0017	266 - 296	EVYL...AIIR
2295.1538	2295.1535	0.0003	297 - 316	SPHL...SLAR
1492.7231	1492.7211	0.0020	317 - 329	SAYW...NSFR
975.5357	975.5349	0.0008	330 - 338	TGTT...NLIR
691.3647	691.3653	-0.0006	339 - 344	SPLYGR
2170.1388	2170.1382	0.0006	345 - 364	EGNT...PIFR
2978.4638	2978.4621	0.0017	365 - 392	TLSY...TISR
537.2912	537.2911	0.0001	393 - 396	SIYR
665.3863	665.3860	0.0003	393 - 397	SIYRK
2841.3900	2841.3933	-0.0033	397 - 423	KSGP...YSHR
2713.2996	2713.2984	0.0012	398 - 423	SGPI...YSHR
1145.5647	1145.5652	-0.0005	424 - 432	LCHA...FLER
528.3017	528.3020	-0.0003	433 - 437	ISGPR

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
1273.6564	1273.6568	-0.0004	438 - 448	IAGT...WTHR
1230.5843	1230.5840	0.0003	449 - 460	SASP...SPSR
983.5802	983.5804	-0.0002	461 - 468	ITQIPWVK
2119.2160	2119.2153	0.0007	461 - 480	ITQI...SVIK
1153.6452	1153.6455	-0.0003	469 - 480	AHTL...SVIK
2325.2455	2325.2441	0.0014	469 - 492	AHTL...ILTR
1189.6101	1189.6092	0.0009	481 - 492	GPGF...ILTR
1076.5298	1076.5284	0.0014	493 - 502	NSMG...GTLR
679.3655	679.3653	0.0002	503 - 508	VTFTGR
1699.9055	1699.9046	0.0009	503 - 516	VTFT...YYIR
1038.5499	1038.5498	0.0001	509 - 516	LPQSYYIR
1082.5652	1082.5621	0.0031	517 - 525	FRYA...VANR
779.3924	779.3926	-0.0002	519 - 525	YASVANR
566.2813	566.2813	0.0000	526 - 530	SGTFR
1469.7206	1469.7191	0.0015	531 - 543	YSQP...SFPK
2628.2534	2628.2530	0.0004	531 - 554	YSQP...LTSR
1176.5445	1176.5445	0.0000	544 - 554	TMDA...LTSR
1724.8899	1724.8886	0.0013	555 - 569	SFAH...TFSR
2044.9717	2044.9742	-0.0025	570 - 586	AQEE...YIDR
2142.0511	2142.0521	-0.0010	587 - 604	IEFI...DLER
1931.0804	1931.0840	-0.0036	605 - 622	AQKV...LGLK
1603.8943	1603.8934	0.0009	608 - 622	VVNA...LGLK
3185.4190	3185.4169	0.0021	623 - 649	TDVT...LDEK
3341.5160	3341.5180	-0.0020	623 - 650	TDVT...DEKR
774.3980	774.3984	-0.0004	661 - 666	RLSDER
618.2972	618.2973	-0.0001	662 - 666	LSDER
1715.8601	1715.8591	0.0010	662 - 675	LSDE...PNFR
1115.5731	1115.5723	0.0008	667 - 675	NLLQ...PNFR
458.2603	458.2601	0.0002	676 - 679	GINR
514.2499	514.2500	-0.0001	680 - 683	QPDR
1551.7422	1551.7417	0.0005	687 - 701	GSTD...DVFK
4133.9082	4133.9092	-0.0010	687 - 722	GSTD...LYQK
2600.1822	2600.1781	0.0041	702 - 722	ENYV...LYQK
831.4703	831.4702	0.0001	723 - 729	IDESKLK
1322.7187	1322.7194	-0.0007	723 - 733	IDES...AYTR
578.3178	578.3176	0.0002	734 - 737	YQLR
1825.9126	1825.9098	0.0028	738 - 752	GYIE...YLIR

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
494.2490	494.2489	0.0001	753 - 756	YNAK
3631.7841	3631.7841	0.0000	753 - 785	YNAK...EPNR
3155.5468	3155.5458	0.0010	757 - 785	HEIV...EPNR
2050.8506	2050.8560	-0.0054	786 - 801	CAPH...CSCR
2480.0419	2480.0420	-0.0001	786 - 805	CAPH...DGEK
1024.5408	1024.5414	-0.0006	836 - 844	IKTQ...GHAR
783.3623	783.3624	-0.0001	838 - 844	TQDGHAR
2111.1646	2111.1626	0.0020	845 - 863	LGNL...ALAR
1592.8764	1592.8774	-0.0010	875 - 887	RETL...IVYK
1436.7744	1436.7762	-0.0018	876 - 887	ETLQ...IVYK
1764.9519	1764.9509	0.0010	876 - 890	ETLQ...KEAK
1969.9379	1969.9381	-0.0002	888 - 904	EAKE...QYDR
1641.7638	1641.7635	0.0003	891 - 904	ESVD...QYDR
1610.8084	1610.8086	-0.0002	905 - 919	LQAD...AADK
1766.9092	1766.9097	-0.0005	905 - 920	LQAD...ADKR
2956.5439	2956.5545	-0.0106	924 - 949	IREA…LEER
2687.3701	2687.3694	0.0007	926 - 949	EAYL...LEER
1302.6612	1302.6608	0.0004	950 - 960	IFTA...YDAR
486.3167	486.3166	0.0001	961 - 964	NIIK
1649.7636	1649.7620	0.0016	965 - 978	NGDF...WNVK
1446.6600	1446.6600	0.0000	979 - 990	GHVE...NNHR
1969.0179	1969.0156	0.0023	991 - 1007	SVLV...QEVR
587.2851	587.2850	0.0001	1008 - 1012	VCPGR
1189.6378	1189.6390	-0.0012	1008 - 1017	VCPG...YILR
620.3646	620.3646	0.0000	1013 - 1017	GYILR
580.3221	580.3221	0.0000	1018 - 1022	VTAYK
3010.3846	3010.3866	-0.0020	1018 - 1043	VTAY...DELK
4094.7379	4094.7422	-0.0043	1044 - 1077	FNNC...YTSR
2807.2318	2807.2310	0.0008	1078 - 1102	NRGY...YEEK
2537.0892	2537.0870	0.0022	1080 - 1102	GYDE...YEEK
640.2822	640.2816	0.0006	1103 - 1107	SYTDR
796.3828	796.3828	0.0000	1103 - 1108	SYTDRR
1316.6012	1316.6003	0.0009	1108 - 1117	RREN...ESNR
1160.4985	1160.4992	-0.0007	1109 - 1117	RENP...ESNR
1004.3982	1004.3981	0.0001	1110 - 1117	ENPCESNR
1600.7768	1600.7773	-0.0005	1118 - 1132	GYGD...YVTK

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
1269.5765	1269.5765	0.0000	1133 - 1142	ELEY...ETDK
4001.9309	4001.9271	0.0038	1133 - 1166	ELEY...LMEE
2750.3644	2750.3612	0.0032	1143 - 1166	VWIE...LMEE

[1] All imported values were rounded to 4 decimal places.
[2] Only experimental masses that matched calculated masses with the highest scores are listed in the table.
[3] The calculated mass is the exact molecular mass calculated from the matched peptide sequence.
[4] The calculated difference = experimental mass – calculated mass.
[5] Position refers to amino acid residues within the predicted MON 95379-produced Cry1Da_7 sequence as depicted in Figure D-2, Panel A.
[6] For peptide matches greater than nine amino acids in length, the first 4 residues and last 4 residues are show separated by three dots (...).

Table D-3. Summary of the Tryptic Masses Identified for the *Bt*-Produced Cry1Da_7 Using LC-MS/MS[1]

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
2262.015	2262.0157	-0.0007	2 - 20	AEIN...SNPK
3201.5143	3201.5182	-0.0039	2 - 28	AEIN...GEER
957.5144	957.5131	0.0013	21 - 28	EIILGEER
763.3872	763.3864	0.0008	88 - 93	IEEFAR
687.3666	687.3664	0.0002	94 - 99	NQAISR
1704.9171	1704.9158	0.0013	94 - 108	NQAI...NLYK
2222.2168	2222.2171	-0.0003	94 - 112	NQAI...VYVR
1035.5602	1035.56	0.0002	100 - 108	LEGL...NLYK
1745.8373	1745.8373	0.0000	113 - 127	AFSD...PALR
2291.0721	2291.064	0.0081	113 - 131	AFSD...EEMR
882.4562	882.4559	0.0003	120 - 127	DPTNPALR
1427.6821	1427.6827	-0.0006	120 - 131	DPTN...EEMR
2063.089	2063.0874	0.0016	132 - 149	IQFN...PLFR
2712.4942	2712.4962	-0.0020	150 - 173	VQNY...SILR
907.44	907.44	0.0000	174 - 181	DVSVFGER
1309.6056	1309.6051	0.0005	182 - 192	WGYD...INNR
2868.3523	2868.3501	0.0022	193 - 216	YSDL...QGLR
1311.6612	1311.6612	0.0000	222 - 231	FLSD...VYNR
1614.8332	1614.8307	0.0025	222 - 233	FLSD...NRFR
1770.932	1770.9318	0.0002	222 - 234	FLSD...RFRR
2379.2964	2379.2951	0.0013	234 - 253	RQLT...YDIR
2223.1949	2223.194	0.0009	235 - 253	QLTI...YDIR
1391.7414	1391.7409	0.0005	254 - 265	TYPI...QLTR
4783.4748	4783.4748	0.0000	254 - 296	TYPI...AIIR
3409.7481	3409.7445	0.0036	266 - 296	EVYL...AIIR
2295.1527	2295.1535	-0.0008	297 - 316	SPHL...SLAR
1492.7235	1492.7211	0.0024	317 - 329	SAYW...NSFR
2450.2467	2450.2455	0.0012	317 - 338	SAYW...NLIR
3123.5982	3123.6003	-0.0021	317 - 344	SAYW...LYGR
975.536	975.5349	0.0011	330 - 338	TGTT...NLIR
691.3659	691.3653	0.0006	339 - 344	SPLYGR
2170.1405	2170.1382	0.0023	345 - 364	EGNT...PIFR
2978.4662	2978.4621	0.0041	365 - 392	TLSY...TISR
3497.7438	3497.7427	0.0011	365 - 396	TLSY...SIYR
3625.8386	3625.8376	0.0010	365 - 397	TLSY...IYRK
2841.3951	2841.3933	0.0018	397 - 423	KSGP...YSHR
2713.3017	2713.2984	0.0033	398 - 423	SGPI...YSHR

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
1145.5655	1145.5652	0.0003	424 - 432	LCHA...FLER
1655.8571	1655.8566	0.0005	424 - 437	LCHA...SGPR
2996.5252	2996.5217	0.0035	433 - 460	ISGP...SPSR
1273.6562	1273.6568	-0.0006	438 - 448	IAGT...WTHR
2486.2324	2486.2302	0.0022	438 - 460	IAGT...SPSR
1230.5847	1230.584	0.0007	449 - 460	SASP...SPSR
2196.1544	2196.1539	0.0005	449 - 468	SASP...PWVK
983.5805	983.5804	0.0001	461 - 468	ITQIPWVK
2119.2152	2119.2153	-0.0001	461 - 480	ITQI...SVIK
3290.8122	3290.814	-0.0018	461 - 492	ITQI...ILTR
1153.6462	1153.6455	0.0007	469 - 480	AHTL...SVIK
2325.2484	2325.2441	0.0043	469 - 492	AHTL...ILTR
1189.6085	1189.6092	-0.0007	481 - 492	GPGF...ILTR
2248.1292	2248.127	0.0022	481 - 502	GPGF...GTLR
2909.4823	2909.4818	0.0005	481 - 508	GPGF...FTGR
1076.5288	1076.5284	0.0004	493 - 502	NSMG...GTLR
1737.8858	1737.8832	0.0026	493 - 508	NSMG...FTGR
679.3647	679.3653	-0.0006	503 - 508	VTFTGR
1699.905	1699.9046	0.0004	503 - 516	VTFT...YYIR
1038.5506	1038.5498	0.0008	509 - 516	LPQSYYIR
2103.1018	2103.1013	0.0005	509 - 525	LPQS...VANR
779.3924	779.3926	-0.0002	519 - 525	YASVANR
1327.6578	1327.6633	-0.0055	519 - 530	YASV...GTFR
1469.7193	1469.7191	0.0002	531 - 543	YSQP...SFPK
2628.2613	2628.253	0.0083	531 - 554	YSQP...LTSR
1176.5456	1176.5445	0.0011	544 - 554	TMDA...LTSR
2883.4187	2883.4225	-0.0038	544 - 569	TMDA...TFSR
1724.8919	1724.8886	0.0033	555 - 569	SFAH...TFSR
2044.9735	2044.9742	-0.0007	570 - 586	AQEE...YIDR
4169.0169	4169.0157	0.0012	570 - 604	AQEE...DLER
2142.0526	2142.0521	0.0005	587 - 604	IEFI...DLER
1931.0842	1931.084	0.0002	605 - 622	AQKV...LGLK
1603.8943	1603.8934	0.0009	608 - 622	VVNA...LGLK
3185.4193	3185.4169	0.0024	623 - 649	TDVT...LDEK
3341.5189	3341.518	0.0009	623 - 650	TDVT...DEKR
3927.8037	3927.8143	-0.0106	623 - 655	TDVT...LSEK
760.4078	760.4079	-0.0001	650 - 655	RELSEK

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
831.4701	831.4702	-0.0001	651 - 657	ELSEKVK
774.3987	774.3984	0.0003	661 - 666	RLSDER
1871.9602	1871.9602	0.0000	661 - 675	RLSD...PNFR
1715.8562	1715.8591	-0.0029	662 - 675	LSDE...PNFR
1115.5729	1115.5723	0.0006	667 - 675	NLLQ...PNFR
954.499	954.4995	-0.0005	676 - 683	GINRQPDR
913.4513	913.4518	-0.0005	680 - 686	QPDRGWR
1950.9428	1950.9436	-0.0008	684 - 701	GWRG...DVFK
1551.7398	1551.7417	-0.0019	687 - 701	GSTD...DVFK
4133.909	4133.9092	-0.0002	687 - 722	GSTD...LYQK
2600.1793	2600.1781	0.0012	702 - 722	ENYV...LYQK
831.4704	831.4702	0.0002	723 - 729	IDESKLK
1322.7204	1322.7194	0.0010	723 - 733	IDES...AYTR
750.4387	750.4388	-0.0001	728 - 733	LKAYTR
1069.5683	1069.5668	0.0015	730 - 737	AYTRYQLR
1825.9098	1825.9098	0.0000	738 - 752	GYIE...YLIR
3631.788	3631.7841	0.0039	753 - 785	YNAK...EPNR
3155.5482	3155.5458	0.0024	757 - 785	HEIV...EPNR
5188.3761	5188.3913	-0.0152	757 - 801	HEIV...CSCR
2050.8556	2050.856	-0.0004	786 - 801	CAPH...CSCR
1024.5416	1024.5414	0.0002	836 - 844	IKTQ...GHAR
783.3623	783.3624	-0.0001	838 - 844	TQDGHAR
2111.1649	2111.1626	0.0023	845 - 863	LGNL...ALAR
1592.8784	1592.8774	0.0010	875 - 887	RETL...IVYK
1921.0559	1921.052	0.0039	875 - 890	RETL...KEAK
1436.7766	1436.7762	0.0004	876 - 887	ETLQ...IVYK
1764.9496	1764.9509	-0.0013	876 - 890	ETLQ...KEAK
3388.7085	3388.7038	0.0047	876 - 904	ETLQ...QYDR
1969.9387	1969.9381	0.0006	888 - 904	EAKE...QYDR
1641.7646	1641.7635	0.0011	891 - 904	ESVD...QYDR
3234.563	3234.5615	0.0015	891 - 919	ESVD...AADK
3390.6575	3390.6626	-0.0051	891 - 920	ESVD...ADKR
1610.8088	1610.8086	0.0002	905 - 919	LQAD...AADK
1766.9087	1766.9097	-0.0010	905 - 920	LQAD...ADKR
2159.1381	2159.1382	-0.0001	905 - 923	LQAD...RVHR
2956.5539	2956.5545	-0.0006	924 - 949	IREA...LEER
2687.3712	2687.3694	0.0018	926 - 949	EAYL...LEER
3972.025	3972.0196	0.0054	926 - 960	EAYL...YDAR

Experimental Mass[2]	Calculated Mass[3]	Difference[4]	Fragment[5]	Sequence[6]
1302.6615	1302.6608	0.0007	950 - 960	IFTA...YDAR
1649.7618	1649.762	-0.0002	965 - 978	NGDF...WNVK
1446.6599	1446.66	-0.0001	979 - 990	GHVE...NNHR
3397.666	3397.6651	0.0009	979 - 1007	GHVE...QEVR
1969.016	1969.0156	0.0004	991 - 1007	SVLV...QEVR
1189.6389	1189.639	-0.0001	1008 - 1017	VCPG...YILR
1182.6756	1182.6761	-0.0005	1013 - 1022	GYIL...TAYK
2968.3751	2968.376	-0.0009	1018 - 1043	VTAY...DELK
2406.0655	2406.0645	0.0010	1023 - 1043	EGYG...DELK
4094.7467	4094.7422	0.0045	1044 - 1077	FNNC...YTSR
2807.2285	2807.231	-0.0025	1078 - 1102	NRGY...YEEK
2537.0816	2537.087	-0.0054	1080 - 1102	GYDE...YEEK
3159.3576	3159.3581	-0.0005	1080 - 1107	GYDE...YTDR
796.3828	796.3828	0.0000	1103 - 1108	SYTDRR
1316.6022	1316.6003	0.0019	1108 - 1117	RREN...ESNR
1160.4985	1160.4992	-0.0007	1109 - 1117	RENP...ESNR
2743.267	2743.266	0.0010	1109 - 1132	RENP...YVTK
1004.3996	1004.3981	0.0015	1110 - 1117	ENPCESNR
2587.1674	2587.1649	0.0025	1110 - 1132	ENPC...YVTK
1600.7776	1600.7773	0.0003	1118 - 1132	GYGD...YVTK
2852.3411	2852.3432	-0.0021	1118 - 1142	GYGD...ETDK
1269.5768	1269.5765	0.0003	1133 - 1142	ELEY...ETDK
4001.9295	4001.9271	0.0024	1133 - 1166	ELEY...LMEE
2750.3631	2750.3612	0.0019	1143 - 1166	VWIE...LMEE

[1] All imported values were rounded to 4 decimal places.
[2] Only experimental masses that matched calculated masses with the highest scores are listed in table.
[3] The calculated mass is the exact molecular mass calculated from the matched peptide sequence.
[4] The calculated difference = experimental mass – calculated mass.
[5] Position refers to amino acid residues within the predicted *Bt*-produced Cry1Da_7 sequence as depicted in Figure D-2, Panel B.
[6] For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are shown separated by dots (…).

(A)

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   1 MAEINNQNQC VPYNCLSNPK EIILGEERLE TGNTVADISL GLINFLYSNF
  51 VPGGGFIVGL LELIWGFIGP SQWDIFLAQI EQLISQRIEE FARNQAISRL
 101 EGLSNLYKVY VRAFSDWEKD PTNPALREEM RIQFNDMNSA LITAIPLFRV
 151 QNYEVALLSV YVQAANLHLS ILRDVSVFGE RWGYDTATIN NRYSDLTSLI
 201 HVYTNHCVDT YNQGLRRLEG RFLSDWIVYN RFRRQLTISV LDIVAFFPNY
 251 DIRTYPIQTA TQLTREVYLD LPFINENLSP AAVYPTFSAA ESAIIRSPHL
 301 VDFLNSFTIY TDSLARSAYW GGHLVNSFRT GTTTNLIRSP LYGREGNTER
 351 PVTITASPSV PIFRTLSYPT GLDNSNPVAG IEGVEFQNTI SRSIYRKSGP
 401 IDSFSELPPQ DASVSPAIGY SHRLCHATFL ERISGPRIAG TVFSWTHRSA
 451 SPTNEVSPSR ITQIPWVKAH TLASGASVIK GPGFTGGDIL TRNSMGELGT
 501 LRVTFTGRLP QSYYIRFRYA SVANRSGTFR YSQPPSYGIS FPKTMDAGEP
 551 LTSRSFAHTT LFTPITFSRA QEEFDLYIQS GVYIDRIEFI PVTATFEAEY
 601 DLERAQKVVN ALFTSTNQLG LKTDVTDYHI DQVSNLVACL SDEFCLDEKR
 651 ELSEKVKHAK RLSDERNLLQ DPNFRGINRQ PDRGWRGSTD ITIQGGDDVF
 701 KENYVTLPGT FDECYPTYLY QKIDESKLKA YTRYQLRGYI EDSQDLEIYL
 751 IRYNAKHEIV NVPGTGSLWP LSVENQIGPC GEPNRCAPHL EWNPDLHCSC
 801 RDGEKCAHHS HHFSLDIDVG CTDLNEDLGV WVIFKIKTQD GHARLGNLEF
 851 LEEKPLLGEA LARVKRAEKK WRDKRETLQL ETTIVYKEAK ESVDALFVNS
 901 QYDRLQADTN IAMIHAADKR VHRIREAYLP ELSVIPGVNA AIFEELEERI
 951 FTAFSLYDAR NIIKNGDFNN GLLCWNVKGH VEVEEQNNHR SVLVIPEWEA
1001 EVSQEVRVCP GRGYILRVTA YKEGYGEGCV TIHEIENNTD ELKFNNCVEE
1051 EVYPNNTVTC INYTATQEEY EGTYTSRNRG YDEAYGNNPS VPADYASVYE
1101 EKSYTDRRRE NPCESNRGYG DYTPLPAGYV TKELEYFPET DKVWIEIGET
1151 EGTFIVDSVE LLLMEE
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(B)

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   1 MAEINNQNQC VPYNCLSNPK EIILGEERLE TGNTVADISL GLINFLYSNF
  51 VPGGGFIVGL LELIWGFIGP SQWDIFLAQI EQLISQRIEE FARNQAISRL
 101 EGLSNLYKVY VRAFSDWEKD PTNPALREEM RIQFNDMNSA LITAIPLFRV
 151 QNYEVALLSV YVQAANLHLS ILRDVSVFGE RWGYDTATIN NRYSDLTSLI
 201 HVYTNHCVDT YNQGLRRLEG RFLSDWIVYN RFRRQLTISV LDIVAFFPNY
 251 DIRTYPIQTA TQLTREVYLD LPFINENLSP AAVYPTFSAA ESAIIRSPHL
 301 VDFLNSFTIY TDSLARSAYW GGHLVNSFRT GTTTNLIRSP LYGREGNTER
 351 PVTITASPSV PIFRTLSYPT GLDNSNPVAG IEGVEFQNTI SRSIYRKSGP
 401 IDSFSELPPQ DASVSPAIGY SHRLCHATFL ERISGPRIAG TVFSWTHRSA
 451 SPTNEVSPSR ITQIPWVKAH TLASGASVIK GPGFTGGDIL TRNSMGELGT
 501 LRVTFTGRLP QSYYIRFRYA SVANRSGTFR YSQPPSYGIS FPKTMDAGEP
 551 LTSRSFAHTT LFTPITFSRA QEEFDLYIQS GVYIDRIEFI PVTATFEAEY
 601 DLERAQKVVN ALFTSTNQLG LKTDVTDYHI DQVSNLVACL SDEFCLDEKR
 651 ELSEKVKHAK RLSDERNLLQ DPNFRGINRQ PDRGWRGSTD ITIQGGDDVF
 701 KENYVTLPGT FDECYPTYLY QKIDESKLKA YTRYQLRGYI EDSQDLEIYL
 751 IRYNAKHEIV NVPGTGSLWP LSVENQIGPC GEPNRCAPHL EWNPDLHCSC
 801 RDGEKCAHHS HHFSLDIDVG CTDLNEDLGV WVIFKIKTQD GHARLGNLEF
 851 LEEKPLLGEA LARVKRAEKK WRDKRETLQL ETTIVYKEAK ESVDALFVNS
 901 QYDRLQADTN IAMIHAADKR VHRIREAYLP ELSVIPGVNA AIFEELEERI
 951 FTAFSLYDAR NIIKNGDFNN GLLCWNVKGH VEVEEQNNHR SVLVIPEWEA
1001 EVSQEVRVCP GRGYILRVTA YKEGYGEGCV TIHEIENNTD ELKFNNCVEE
1051 EVYPNNTVTC INYTATQEEY EGTYTSRNRG YDEAYGNNPS VPADYASVYE
1101 EKSYTDRRRE NPCESNRGYG DYTPLPAGYV TKELEYFPET DKVWIEIGET
1151 EGTFIVDSVE LLLMEE
```

Figure D-2. Peptide Map of the MON 95379-Produced Cry1Da_7 and *Bt*-Produced Cry1Da_7

(A). The amino acid sequence of the MON 95379-produced Cry1Da_7 protein was deduced from the *cry1Da 7* gene present in MON 95379. Boxed regions correspond to peptides that were identified from the MON 95379-produced Cry1Da_7 protein sample using LC-MS/MS. In total, 89% coverage (1049 out of 1166 amino acids) of the expected protein sequence was covered by the identified peptides. Gray highlighted regions correspond to the receptor binding domain (see Appendix L).

(B). The amino acid sequence of the *Bt*-produced Cry1Da_7 protein was deduced from the *cry1Da_7* gene that is contained on the expression plasmid pMON417133. Boxed regions correspond to peptides that were identified from the *Bt*-produced Cry1Da_7 protein sample using LC-MS/MS. In total, 89% coverage (1049 out of 1166 amino acids) of the expected protein sequence was covered by the identified peptides. Gray highlighted regions correspond to the receptor binding domain (see Appendix L).

D.1.4. Results of Western Blot Analysis of the Cry1Da_7 Protein Isolated from the Grain of MON 95379 and Immunoreactivity Comparison to *Bt*-produced Cry1Da_7 Protein

Western blot analysis was conducted using goat anti-Cry1Da_7 polyclonal antibody as additional means to confirm the identity of the Cry1Da_7 protein isolated from the grain of MON 95379 and to assess the equivalence of the immunoreactivity of the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins.

The results showed that immunoreactive bands with the same electrophoretic mobility were present in all lanes loaded with the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins (Figure D-3). For each amount loaded, comparable signal intensity was observed between the MON 95379-produced and *Bt*-produced Cry1Da_7 protein bands. As expected, the signal intensity increased with increasing load amounts of the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins, thus, supporting identification of MON 95379-produced Cry1Da_7 protein.

To compare the immunoreactivity of the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins, densitometric analysis was conducted on the bands that migrated at the expected apparent MW for Cry1Da_7 proteins (~ 130 kDa). The signal intensity (reported in OD) of the band of interest in lanes loaded with MON 95379-produced and *Bt*-produced Cry1Da_7 proteins was measured (Table D-4). Because the mean signal intensity of the MON 95379-produced Cry1Da_7 protein was within 35% of the mean signal intensity of the *Bt*-produced Cry1Da_7 protein, the MON 95379-produced Cry1Da_7 and *Bt*-produced Cry1Da_7 proteins were determined to have equivalent immunoreactivity.

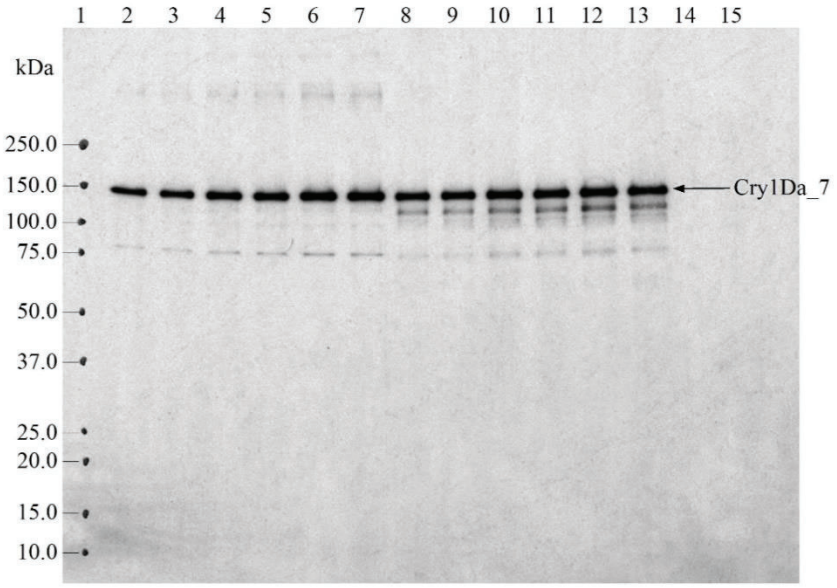

Figure D-3. Western Blot Analysis and Immunoreactivity of MON 95379-Produced and Bt-Produced Cry1Da_7 Proteins

Aliquots of the MON 95379-produced and Bt-produced Cry1Da_7 proteins were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. Proteins were detected using goat anti-Cry1Da_7 polyclonal antibodies as the primary antibodies. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system. The 3-minute exposure is shown. The approximate MW (kDa) of the standards are shown on the left. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Bt-produced Cry1Da_7	10
3	Bt-produced Cry1Da_7	10
4	Bt-produced Cry1Da_7	15
5	Bt-produced Cry1Da_7	15
6	Bt-produced Cry1Da_7	20
7	Bt-produced Cry1Da_7	20
8	MON 95379-produced Cry1Da_7	10
9	MON 95379-produced Cry1Da_7	10
10	MON 95379-produced Cry1Da_7	15
11	MON 95379-produced Cry1Da_7	15
12	MON 95379-produced Cry1Da_7	20
13	MON 95379-produced Cry1Da_7	20
14	Blank	-
15	Blank	-

Table D-4. Immunoreactivity of the MON 95379-Produced and *Bt*-Produced Cry1Da_7 Proteins

Mean Signal Intensity from MON 95379-Produced Cry1Da_7 [1] (OD)	Mean Signal Intensity from *Bt*-Produced Cry1Da_7 [1] (OD)	Acceptance Limits[2] (OD)
1260.28	1175.99	764.39 – 1587.59

[1] Each value represents the mean of six values (n = 6).

[2] The acceptance limits are for the MON 95379-produced Cry1Da_7 protein and are based on the interval between -35% (1175.99 x 0.65 = 764.39) and +35 % (1175.99 x 1.35 = 1587.59) of the mean of the *Bt*-produced Cry1Da_7 signal intensity across all loads.

D.1.5. Results of the Cry1Da_7 Protein Molecular Weights and Purity Analysis

For apparent MW and purity determination, the MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7 proteins were subjected to SDS-PAGE. Following electrophoresis, the gel was stained with Brilliant Blue G-Colloidal stain and analyzed by densitometry. The MON 95379-produced Cry1Da_7 protein (Figure D-4, lanes 3-8) migrated to the same position on the gel as the *Bt*-produced Cry1Da_7 protein (Figure D-4, lane 2) and the apparent MW was calculated to be 132.1 kDa (Table D-5). Because the experimentally determined apparent MW of the MON 95379-produced Cry1Da_7 protein was within the acceptance limits for equivalence (Table D-6), the MON 95379-produced Cry1Da_7 and *Bt*-produced Cry1Da_7 proteins were determined to have equivalent apparent molecular weights.

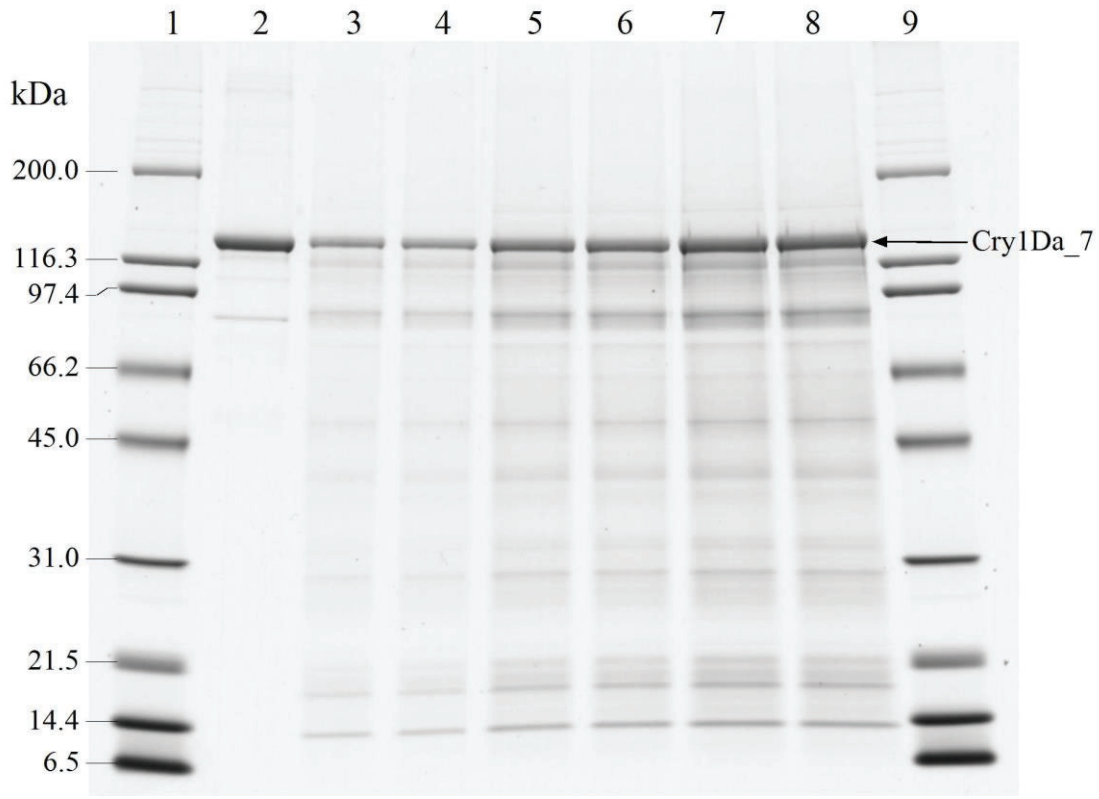

Figure D-4. Purity and Apparent Molecular Weight Analysis of the MON 95379-Produced Cry1Da_7 Proteins

Aliquots of the MON 95379-produced and the *Bt*-produced Cry1Da_7 proteins were subjected to SDS-PAGE and the gel was stained with Brilliant Blue G-Colloidal stain. The MWs (kDa) are shown on the left and correspond to the standards loaded in lanes 1 and 9. Lane 10 was cropped from the image. The intact Cry1Da_7 protein is indicated with an arrow in the image. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standard	5.0
2	*Bt*-produced Cry1Da_7	1.0
3	MON 95379-produced Cry1Da_7	1.0
4	MON 95379-produced Cry1Da_7	1.0
5	MON 95379-produced Cry1Da_7	2.0
6	MON 95379-produced Cry1Da_7	2.0
7	MON 95379-produced Cry1Da_7	3.0
8	MON 95379-produced Cry1Da_7	3.0
9	Broad Range MW Standard	5.0
10	Blank	

Table D-5. Apparent Molecular Weight and Purity Analysis of the MON 95379-Produced Cry1Da_7 Protein

	Apparent MW[1] (kDa)	Purity[2] (%)
Average (n = 6)	132.1	52

[1]Final MW was rounded to one decimal place.
[2]Average % purity was rounded to the nearest whole number.

Table D-6. Apparent Molecular Weight Comparison Between the MON 95379-Produced Cry1Da_7 and *Bt*-Produced Cry1Da_7 Proteins

Apparent MW of MON 95379-Produced Cry1Da_7 Protein (kDa)	Apparent MW of *Bt*-Produced Cry1Da_7 Protein[1] (kDa)	Acceptance Limits[2] (kDa)
132.1	126.5	119.9 – 133.1

[1] As reported on the COA of the *Bt*-produced Cry1Da_7 protein (lot 7799).
[2] Data obtained for the *Bt*-produced Cry1Da_7 protein were used to generate the prediction interval. Values in this column represent a 95% prediction interval developed from eight individual assays with *Bt*-produced Cry1Da_7 protein (Section D.2.3.6. and Table D-8).

D.1.6. Cry1Da_7 Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether the Cry1Da_7 protein was glycosylated when expressed in the maize grain of MON 95379, the MON 95379-produced Cry1Da_7 protein was analyzed using an ECL™ glycoprotein detection method. Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins, the *Bt*-produced Cry1Da_7 protein was also analyzed.

A clear glycosylation signal was observed at the expected molecular weight (~80 kDa) in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure D-5, Panel A). In contrast, no glycosylation signal was observed in the lanes containing the *Bt*-produced Cry1Da_7 protein or MON 95379-produced Cry1Da_7 protein (Figure D-5, Panel A).

To confirm that MON 95379-produced Cry1Da_7 and *Bt*-produced Cry1Da_7 proteins were appropriately loaded for glycosylation analysis, a second membrane with identical loadings and transfer time was stained with Coomassie Blue R250 for protein detection. Both the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins were detected (Figure D-5, Panel B). These data indicate that the glycosylation status of MON 95379-produced Cry1Da_7 protein is equivalent to that of the *Bt*-produced Cry1Da_7 protein and that neither is glycosylated.

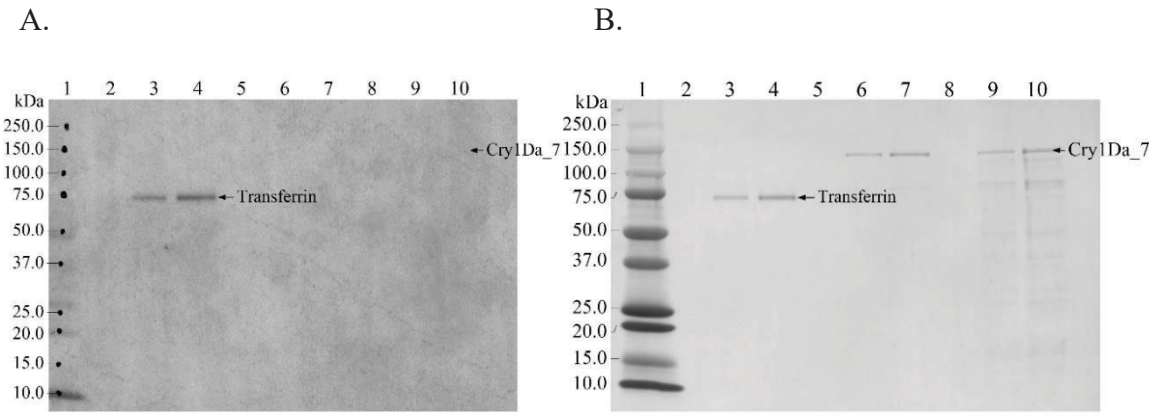

Figure D-5. Glycosylation Analysis of the MON 95379-Produced and *Bt*-Produced Cry1Da_7 Proteins

Aliquots of the transferrin (positive control), *Bt*-produced Cry1Da_7 and MON 95379-produced Cry1Da_7 proteins were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. The MWs (kDa) correspond to the Precision Plus Protein™ Standards. The arrows show the expected migration of the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins and transferrin. (A) Where present, the labeled carbohydrate moieties were detected by using ECL reagents and exposure to Hyperfilm™. The 4-minute exposure is shown. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Blank	-
3	Transferrin (positive control)	100
4	Transferrin (positive control)	200
5	Blank	-
6	*Bt*-produced Cry1Da_7	100
7	*Bt*-produced Cry1Da_7	200
8	Blank	-
9	MON 95379-produced Cry1Da_7	100
10	MON 95379-produced Cry1Da_7	200

D.1.7. Cry1Da_7 Functional Activity

The MON 95379-produced Cry1Da_7 and *Bt*-produced Cry1Da_7 proteins were considered to have equivalent functional activity if the activities of both proteins (EC_{50} values) were within acceptance limits of 0.076 to 0.293 µg of Cry1Da_7 protein/mL diet, which is the prediction interval calculated from a data set of *Bt*-produced Cry1Da_7 protein activity.

The experimentally determined functional activity for the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins are presented in Table D-7. The functional activities of MON 95379-produced and *Bt*-produced Cry1Da_7 proteins were 0.17 and 0.12 µg of Cry1Da_7 protein/mL diet, respectively. Because the functional activities of MON 95379-produced and *Bt*-produced Cry1Da_7 proteins fall within the preset acceptance limits (Table D-7), the MON 95379-produced Cry1Da_7 protein was considered to have equivalent functional activity to that of the *Bt*-produced Cry1Da_7 protein.

Table D-7. Functional Activity of MON 95379-Produced and *Bt*-Produced Cry1Da_7 Proteins

Replicates	MON 95379-Produced Cry1Da_7 EC_{50} [1] (µg protein/mL diet)	*Bt*-Produced Cry1Da_7 EC_{50} [1] (µg protein/mL diet)	EC_{50} Acceptance Limits[2] (µg protein/mL diet)
1	0.20	0.11	0.076 – 0.293
2	0.14	0.076	
3	0.17	0.16	
Mean	0.17	0.12	-

[1] Value refers to mean EC_{50} calculated based on three independent assays (n = 3).

[2] Data obtained for the *Bt*-produced Cry1Da_7 proteins were used to generate a prediction interval for setting the acceptance limits for EC_{50} values of 3 future independent assays. Values in this column represent a 95% prediction interval developed from 24 individual bioassays with *Bt*-produced Cry1Da_7 protein (Section D.2.3.6. and Table D-9).

D.1.8. Cry1Da_7 Protein Identity and Equivalence Conclusion

The MON 95379-produced Cry1Da_7 protein purified from MON 95379 grain was characterized, and a comparison of the physicochemical and functional properties between the MON 95379-produced and the *Bt*-produced Cry1Da_7 proteins was conducted following a panel of analytical tests: 1) N-terminal sequence analysis established the same identity for the MON 95379-produced and the *Bt*-produced Cry1Da_7 proteins; 2) Nano LC-MS/MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the *cry1Da_7* gene product present in MON 95379; 3) the MON 95379-produced and the *Bt*-produced Cry1Da_7 proteins were both detected on a western blot probed with antibodies specific for Cry1Da_7 protein and the immunoreactive properties of both proteins was shown to be equivalent; 4) the electrophoretic mobility and apparent molecular weight of the MON 95379-produced and the *Bt*-produced Cry1Da_7 proteins were shown to be equivalent; 5) the glycosylation status of MON 95379-produced and the *Bt*-produced Cry1Da_7 proteins was determined to be equivalent; and 6) the functional activity of the MON 95379-produced and the *Bt*-produced Cry1Da_7 was demonstrated to be equivalent. These results demonstrate that the MON 95379-produced and the *Bt*-produced Cry1Da_7 protein are equivalent.

The ability of the Cry1Da_7 to bind to its target receptor is dependent on the confirmation of the Cry1Da_7 receptor binding domain. Protein structure, or confirmation, is determined by the specific sequence of amino acids that comprise the different structural and functional elements. It can inferred that once properly folded, proteins that possess equivalent amino acid sequences would have equivalent functional activity or receptor binding. The MON 95379- and *Bt*-produced Cry1Da_7 proteins possess equivalent amino sequences, and thereby equivalent receptor binding domains, as evidenced by the very high amino acid sequence similarity seen by mass fingerprint analysis where >90% coverage was observed. The equivalency of the protein sequences was also confirmed indirectly through sequencing the of genes of the expressed MON 95379- and *Bt*-produced Cry1Da_7 proteins. Finally, the equivalent functional activity of the protein isolated from both sources indicates that the confirmation of the receptor binding domains in both proteins are equivalent.

Taken together, the equivalency data provided in support of MON 95379 Cry1Da_7 confirms that the *Bt*-produced Cry1Da_7 protein is appropriate surrogate for use in the evaluation of the safety of the MON 95379-produced Cry1Da_7 protein.

D.2. Materials and Methods for Characterization of Cry1Da_7 Protein Produced in MON 95379

D.2.1. Materials

The test substance was the MON 95379-produced Cry1Da_7 protein (lot 8206) purified from grain of MON 95379 (lot 11479279). The MON 95379-produced Cry1Da_7 protein was stored in a -80 ºC freezer in a buffer containing 25 mM sodium carbonate, pH 10.5.

The reference substance was the *Bt*-produced Cry1Da_7 (lot 7799, historical Orion lot 11479575). The Cry1Da_7 reference substance was generated from cell paste produced by fermentation of *Bt* containing the pMON417133 expression plasmid. The DNA sequence encoding Cry1Da_7 protein that is contained on the expression plasmid was confirmed both prior to and following fermentation.

D.2.2. Protein Purification

The plant-produced Cry1Da_7 protein used in this equivalence study was purified from the grain of MON 95379 maize. The purification procedure was not performed under a GLP plan; however, all procedures were documented in ELN and, where applicable, SOPs were followed. A unique purification process was developed to combine both Cry1B.868 and Cry1Da_7 isolations into one single process, as they share similar physicochemical properties. Both Cry1B.868 and Cry1Da_7 were purified from an extract of ground grain using a combination of anion-exchange chromatography and immunoaffinity chromatography (IAC). A detailed description of the purification procedure specific for Cry1Da_7 protein was archived under lot 8206 in the Bayer Regulatory Archives and a brief purification procedure was described below. All purification steps were performed at ~4 °C, except where specifically stated.

Grain of MON 95379 was ground to fine powder in the presence of dry ice and stored at -80 °C until use. A total of ~47 kg of ground powder was processed by 5 different runs. For each run, ~ 10 kg of ground powder was mixed with 100 L of Extraction Buffer (50 mM Na_2CO_3, 5 mM DTT, 2 mM EDTA, 2 mM Benzamine-HCl, 0.5 mM AEBSF, 0.15 M NaCl, pH ~11.3) at 4°C for ~2 hr. The slurry was clarified by centrifugation at 13,500 x g for 1 h at ~4°C, and about 90 L supernatants were collected, filtered, and loaded onto a ~3 L Q Sepharose Fast Flow (QFF; GE Healthcare, Piscataway, NJ) column (15 cm x 20 cm) after equilibrated with Binding Buffer A for QFF (50 mM Carbonate/Bicarbonate Buffer, 5 mM DTT, 2 mM EDTA, 2 mM benzamidine-HCl, 0.1 mM AEBSF, pH 9.0). Proteins were eluted with a linear salt gradient that increased from 0.1 M to 1.0 M NaCl in Elution Buffer B for QFF (50 mM Carbonate/Bicarbonate Buffer, 5 mM DTT, 2 mM EDTA, 2 mM benzamidine-HCl, 0.1 mM AEBSF, 1 M NaCl, pH 9.0) over a volume of ~30 L (~10 x column volume) after the column was washed with 10 % Elution Buffer B for QFF. Fractions containing both Cry1B.868 and Cry1Da_7, identified by western blot analysis, were pooled to a final volume of ~7 L. This pooled sample was concentrated and buffer-exchanged to Wash and Binding Buffer A for IAC (50 mM Carbonate/Bicarbonate Buffer, 2 mM EDTA, 2 mM benzamidine-HCl, 0.15 M NaCl, pH 9.0), using an ultrafiltration system sequentially equipped with a 30,000 NMWC Hollow Fiber Cartridge (8400 cm2 surface area) (GE Healthcare). About 1 L of final sample was collected and stored on wet ice for next step IAC.

For 1st, 2nd, 4th, and 5th production runs, the following steps were conducted for IAC. About 1 L of protein sample collected after buffer exchange was loaded onto a ~75-ml of MabSelect Protein A resin (GE healthcare, Cat #: 17-5199-02) cross-linked with ~150 mg of anti-Cry1Da_7 monoclonal antibody (mAb) by BS^3 following the manufacturer's instruction and equilibrated with Wash and Binding Buffer for IAC. Cry1Da_7 protein was bound by the resin, whereas Cry1B.868 was not bound by the resin (confirmed by western blot analysis). The flow through samples from 1st and 2nd production runs were collected and used for the further purification of Cry1B.868

protein, but the flow through samples from 4th and 5th production runs were discarded since a sufficient amount of Cry1B.868 protein was already purified. Cry1Da_7 protein was eluted from the immunoaffinity column by Polyol Buffer (50 mM Tris, pH 8.0, 0.75 M $(NH_4)_2SO_4$, 40% propylene glycol, 2 mM EDTA, 2 mM Benzamidine-HCl) after the column was washed with Wash Buffer (50 mM Tris, pH 8.0, 0.75 M $(NH_4)_2SO_4$, 2 mM EDTA, 2 mM Benzamidine-HCl). Fractions containing Cry1Da_7 protein were identified by SDS-PAGE and western blot analysis, pooled together, and stored at -80°C for final concentration and buffer exchange.

For 3rd production run, the following steps were conducted for IAC. About 1 L of final sample collected after buffer exchange was sequentially loaded onto the same anti-Cry1Da_7 mAb column described earlier and a ~100 ml anti-Cry1B.868 mAb column (two mAb columns were tandem-connected). Cry1Da_7 protein was bound by the anti-Cry1Da_7 mAb resin, whereas Cry1B.868 protein was bound by the anti-Cry1B.868 mAb resin (confirmed by western blot analysis). After sample load was completed, both columns were disconnected. The anti-Cry1Da_7 mAb resin/column bound with Cry1Da_7 protein was used for next step Cry1Da_7 protein purification following the steps described earlier. The anti-Cry1B.868 mAb resin/column bound with Cry1B.868 protein was used for Cry1B.868 protein purification later.

After 5 individual runs were completed, each final pooled protein sample (~200 ml) was concentrated and run buffer exchange to Storage Buffer for Cry1Da_7 (25 mM Sodium Carbonate, pH 10.5) using 4 x 15 ml Amicon Ultracel-30K centrifugal concentration device (Millipore, Tullagreen, Ireland), and further concentrated using 4 x 0.5 ml Amicon Ultracel-30K centrifugal concentration device (Millipore) at 4°C, to a final volume of 0.4 ml. All final protein samples after being concentrated and buffer exchanged were pooled together and that was the final Cry1Da-7 sample. Buffer composition of the final sample was the same as Storage Buffer for Cry1Da_7. This Cry1Da_7 protein purified from the grain of MON 95379 was aliquoted, assigned lot 8206, and stored in a -80 °C freezer.

D.2.3. Methods for Characterization

D.2.3.1. N-Terminal Sequencing/Mass Fingerprint Analysis

The test substance was analyzed for N-terminal amino acid sequence and peptide mass fingerprint by LC-MS/MS. An aliquot of the test substance was subjected to SDS-PAGE and proteins were visualized on the gel with InstantBlue™ (Expedeon, Cambridgeshire, UK) staining followed by de-staining with Milli-Q water. Gel bands containing Cry1Da_7 (~1 µg in each gel band) were excised, de-stained, incubated in ~200 µl of 100 mM ammonium bicarbonate for 30 min at room temperature (RT) for each gel band. The protein in each gel band was reduced by incubation in 100 µl of 10 mM DTT at ~37°C for 1 hr, alkylated by incubation in the dark for 30 min at RT with 100 µl of 20 mM iodoacetamide, and incubated in 200 µl of 25 mM ammonium bicarbonate for ~15 min with agitation at RT for three times. Each gel band was dried down, rehydrated in 20 µl of 20 µg/ml trypsin protease solution and incubated at RT for 1 hr to allow the solution to diffuse into the gel band. After the excess liquid was removed, 40 µl of 25 mM ammonium bicarbonate/10% acetonitrile solution was added to each gel band and the reaction was incubated at 37°C for ~17 hr. After each tube was sonicated for 6 min, the supernatant was collected. The

digest was extracted with 30 µl of 70% acetonitrile/0.1% formic acid (FA) twice. The samples were combined and completely dried using vacuum centrifugation and then solubilized in 10 µl of 0.1 % FA for LC-MS/MS analysis.

The LC-MS/MS data acquisition was performed on a Dionex 3000 Ultimate nanoLC- system (Dionex) interfaced to an orbitrap fusion mass spectrometer (Thermo Scientific), which is equipped with a nano-ESI (electrospray ionization) source. The sample was loaded and separated online using an Acclaim PepMap100 C18 nano column (75 µm id × 150 mm, 2 µm, 100 Å, Dionex). The separation of the digests was achieved at a 300 nl/min flow rate using an acetonitrile gradient as follows: 0-4 min sustaining 2% solvent B, 4-80 min ramping solvent B 2-40%, 80-85 min ramping solvent B 40-90%. The column was washed/equilibrated between injections as follows: 85-90 min maintaining solvent B at 90%, 90-90.1 min decreasing solvent B 90-2%, and 90.1-100 min maintaining solvent B at 2%. Solvent A was 0.1% FA in water. Solvent B was 0.1% FA in acetonitrile.

The orbitrap fusion mass spectrometer was operated with two scan events. The first scan event was a full Fourier transform mass spectrometry scan with a range of m/z from 200 to 1800 and a mass resolution of 120,000 at m/z of 200. The second scan event was a tandem mass spectrometry scan of fragments from collision induced dissociation (CID) of precursor ions from the first scan event with an isolation width of 2.0 m/z.

The LC-MS/MS dataset were analyzed using Mascot (version 2.5, Matrix Science). MS/MS spectra were searched against the theoretical Cry1Da_7 protein sequence. The mass tolerances of MS1 mass and MS/MS mass were set as 5 ppm and 0.5 Da, respectively. Carboxymethylation of cysteine was set as a fixed modification. Methionine oxidation and N-terminal amino acid acetylation were set as variable modifications. A maximum of two missed cleavages for trypsin were allowed. The mass coverage was accepted if it is greater than 40% of the protein sequence.

D.2.3.2. Western Blot Analysis-Immunoreactivity

Western blot analysis was performed as follows to confirm the identity of the MON 95379-produced Cry1Da_7 protein and to compare the immunoreactivity of the MON 95379-produced and *Bt*-produced Cry1Da_7 protein. MON 95379-produced and *Bt*-produced Cry1Da_7 proteins were diluted to 2 ng/µl (purity-corrected intact Cry1Da_7 protein concentration) in 5 × LB and 6 M urea to obtain a final concentration of 1 × LB and heated to 100°C for 5 min. Three amounts (~10, ~15, and ~20 ng) of the intact test substance (total protein concentration × purity of the intact Cry1Da_7 protein) and the intact reference substance (total protein concentration × purity of the intact Cry1Da_7 protein) were loaded in duplicate onto a pre-cast Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight standards (Precision Plus Protein Standards™, Bio-Rad) were loaded on the gel for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad).

The western blotting procedure was performed using an iBind™ Western System apparatus (Life Technologies, Grand Island, NY). The membrane was blocked with 1 × iBind™ Solution (Life

Technologies) and incubated with goat anti-Cry1Da_7 polyclonal antibody (lot G1496) at a dilution of 1:1000 in 1 × iBind™ Solution. After washing with 1 × iBind™ Solution, the membrane was next incubated with a horseradish peroxidase (HRP)-conjugated anti-goat IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:2000 in 1 × iBind™ Solution and washed again with 1 × iBind™ Solution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare) and exposed to Hyperfilm™ ECL high performance chemiluminescence film (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica, Tokyo, Japan).

Image acquisition and quantification of the bands on the film were performed on a Bio-Rad GS-900 densitometer with the supplied Image Lab 5.0 Security Edition software using the volume tool. The signal intensities of the immunoreactive bands migrating at the expected position for the Cry1Da_7 protein were quantified as adjusted volume values. The immunoreactivity was reported in OD, which equals to the total sum of the intensities of the pixels inside the volume boundary.

D.2.3.3. Apparent Molecular Weight and Purity Determination by SDS-PAGE

MON 95379-produced and *Bt*-produced Cry1Da_7 proteins were diluted in 5 × loading buffer [LB, 0.31 M Tris-Cl, pH 7.5, 10% SDS, 50% glycerol, 25% (v/v) 2-mercaptoethanol, 0.025% (w/v) Bromophenol blue] and 6 M urea and heated to 95-105°C for 3-5 min. The MON 95379-produced Cry1Da_7 protein was loaded in duplicate at ~1.0, ~2.0, and ~3.0 µg based on total protein concentration, onto a Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen, Carlsbad, CA) in lanes 3, 4, 5, 6, 7 and 8. The *Bt*-produced Cry1Da_7 protein was loaded at ~1.0 µg total protein in lane 2. Broad Range Molecular Weight Standards (Bio-Rad) were prepared and loaded in lanes 1 and 9 on the gel. Following electrophoresis at a constant voltage, proteins were briefly fixed in 40% (v/v) methanol, 7% (v/v) acetic acid and stained for 18 ± 2 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO). Gels were briefly destained in 10% (v/v) acetic acid, 25% (v/v) methanol followed by 8 ± 2 hr in 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-900 densitometer supplied with Image Lab 5.0 Security Edition software. The molecular weight of the test substance was calculated based on the mean of the molecular weight of the major band of Cry1Da_7 (~130 kDa) in all six lanes containing the test substance. The purity of the test substance was calculated based on multiple stained bands corresponding to the Cry1Da_7 protein including a major band (~130 kDa) and three minor bands (~120, ~110, and ~90 kDa) recognized by western blot analysis. The purity was reported as an average of all 6 lanes containing the MON 95379-produced Cry1Da_7 protein.

D.2.3.4. Glycosylation Analysis

An ECL glycoprotein detection method (GE Healthcare) was used for glycoprotein detection. The MON 95379-produced Cry1Da_7 protein and the *Bt*-produced Cry1Da_7 protein were diluted in 5 × LB and 6 M urea while a positive control, transferrin (Sigma) was diluted in 5 × LB and water and then heated to 100.6°C for 5 min. Two amounts (~ 100 ng and ~ 200 ng) of the intact MON 95379-produced Cry1Da_7 protein (purity-corrected), the intact *Bt*-produced Cry1Da_7 protein (purity-corrected), and the positive control were loaded onto a pre-cast Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight markers (Precision Plus

Protein™ Standards, Bio-Rad) were also loaded for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

Glycosylation analysis was performed on the PVDF membrane at room temperature using the ECL Glycoprotein Detection Module (GE Healthcare) as directed by the manufacturer. The labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based detection using ECL reagents (GE Healthcare) and exposure to Hyperfilm™ (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta). An identical gel was run and electrotransferred to a PVDF membrane in parallel. Proteins were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) and then destained with equivalent chemical reagents to 1× Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad). After washing with water, the blot was scanned using a Bio-Rad GS-900 densitometer with the supplied Image Lab 5.0 Security Edition software.

D.2.3.5. Functional Activity

Insects. Fall armyworm (FAW, *Spodoptera frugiperda*) is an appropriate test system due to its susceptibility to the Cry1Da_7 protein. FAW were obtained from Bayer (Union City, TN). Insect eggs were incubated at temperatures ranging from 10° C to 27° C, to achieve the desired hatch time.

Bioassays. FAW larvae were used to measure the functional activity of the MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7 in accordance with ME-0044-04. The MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7 substances were analyzed concurrently in each bioassay replicate. Bioassay replicates were initiated on different days, each using a separate batch of insects. Bioassay replicate one consisted of a series of six dilutions yielding a dose series with a two-fold separation factor ranging from 0.05 – 1.6 µg Cry1Da_7/ml diet for the MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7, as well as, an assay (buffer) control. Bioassay replicates two and three consisted of a series of six dilutions yielding a dose series with a two-fold separation factor ranging from 0.025 – 0.8 µg Cry1Da_7/ml diet for the MON 95379-produced Cry1Da_7 and the *Bt*-produced Cry1Da_7, as well as, an assay (buffer) control. Dosing solutions (5.0 ml) were prepared by diluting the protein sample with purified water and incorporating the dilution into an agar-based FAW diet (Southland, Village Lake, AR) to a final volume of 25 ml. Each diet mixture was dispensed in 1 mL aliquots into 16 wells of a 128-well tray (Frontier Agricultural Services). Each well was targeted to be infested with a single FAW larva (≤ 30 hours after first observation of hatching). Larvae were allowed to feed for a period of seven days in an environmental chamber programmed at 27 ºC, 60% relative humidity, and a lighting regime of 14 hours light: 10 hours dark. The number of FAW larvae infested, the number of surviving larvae, and the combined mass of the surviving larvae were recorded for each treatment and concentration level at the end of the seven-day incubation period.

The functional activity of the test substance and reference substance were characterized using a fall armyworm insect bioassay. The biological activity of the test substance and reference substance were measured as a 50% effective concentration (i.e. EC_{50} value). The EC_{50} value is

defined as the concentration that results in 50% growth inhibition. Logistic regression was used to estimate the EC_{50} values for growth inhibition for each bioassay. The insect bioassay was considered acceptable if less than or equal to 20% mortality was observed for the vehicle control and if >50% growth inhibition was observed relative to the vehicle control in the highest tested protein concentration level.

D.2.3.6. Prediction Intervals as Acceptance Criteria

Acceptance criteria (acceptance limits) based on prediction intervals were used to assess the equivalence of the MON 95379-produced and *Bt*-produced Cry1Da_7 proteins for apparent MW and functional activity. A prediction interval is an estimate of an interval in which a randomly selected future observation from a population will fall, with a certain degree of confidence, given what has already been observed; *i.e.*, prediction intervals are generated based on statistical analysis of existing data.

The source of the data used to generate the prediction intervals for apparent MW and functional activity for Cry1Da_7 protein equivalence assessment are provided in Table D-8 and Table D-9, respectively. The data used were generated under GLP guidelines and included apparent MW and functional activity estimates for the reference *Bt*-produced Cry1Da_7 protein.

The two-sided 95% prediction interval (PI) for one future assay was calculated using JMP software (version 9.4, SAS Institute, Inc., Cary, NC) according to the guidance document of Ramírez (2009).

Table D-8. Individual Assay Data and 95% Prediction Interval Generated for the Apparent MW of Cry1Da_7 Protein for One Future Assay

Assay Number[1]	Apparent MW of Cry1Da_7 Protein (kDa)[2]
1	128.645
2	127.876
3	127.103
4	129.681
5	123.739
6	124.134
7	122.535
8	128.281
Mean	126.5
Standard Deviation	2.65
95% Prediction Interval	**119.9 – 133.1**
99% Prediction Interval	116.7 – 136.3

[1] Assay 1-8 represents the MW data from the initial characterization of *Bt*-produced Cry1Da_7 (lot 7799).
[2] The values in the table represent the mean of six (n = 6) data points within each assay. With 95% confidence, the mean of the 6 data points from the next single assay of the population will fall within the stated interval. Mean and prediction interval values rounded to one decimal point.

Table D-9. Estimated EC_{50} Values from 24 Dose-Response Diet Incorporation Insect Bioassays of the Cry1Da_7 Protein

Assay Number[1]	Functional Activity of Cry1Da_7 Protein (U/mg)
1	0.135
2	0.112
3	0.169
4	0.127
5	0.135
6	0.141
7	0.132
8	0.167
9	0.250
10	0.156
11	0.184
12	0.125
13	0.200
14	0.118
15	0.092
16	0.165
17	0.179
18	0.095
19	0.197
20	0.194
21	0.226
22	0.141
23	0.122
24	0.141
Mean	0.149
95% Prediction Interval	**0.076 – 0.293**
99% Prediction Interval	0.064 – 0.351

[1] Assay 1-24 represents the functional activity from the characterization of *Bt*-produced Cry1Da_7 protein (lot 7799).

References for Appendix D

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Appendix E: Materials and Methods Used for the Analysis of the Levels of Cry1B.868 and Cry1Da_7 Proteins Expressed in MON 95379

E.1. Materials

OSL1, OSR1, OSL4, silk, pollen, forage, forage-root and grain tissue samples from five sites in MON 95379 were harvested from United States Field sites during the 2018 growing season from starting seed lot 11479279. ELISA Cry1B.868 (7565) and Cry1Da_7 (7799) was used as the analytical reference standard.

E.2. Characterization of the Materials

The identity of the test substance was confirmed by analysis of the starting seed DNA by an event-specific polymerase chain reaction method.

E.3. Field Design and Tissue Collection

The test substance was planted in four replicated plots at five sites using randomized complete block design.

E.4. Tissue Processing and Protein Extraction

The Cry1B.868 and Cry1Da_7 proteins were extracted from tissue samples as described in Tables E-1 and E-2, respectively.

Table E-1. Cry1B.868 Extraction Parameter [1]

Sample Type	Target Tissue to Buffer Ratio	Extraction Buffer
OSL1	1:100	100 mM Sodium Carbonate Extraction Solution[2]
OSR1	1:100	100 mM Sodium Carbonate Extraction Solution[2]
OSL4	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Silk	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Pollen	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Forage	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Forage Root	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Grain	1:100	100 mM Sodium Carbonate Extraction Solution[2]

[1] Cry1B.868 protein was extracted by adding the appropriate volume of extraction buffer, metal beads, and shaking in a Geno Grinder (SPEX Inc., Metuchen, NJ). The extracted samples were clarified by centrifugation

[2] 100 mM Na_2CO_3/ 0.2% (v/v) Tween 20, 0.2% (v/v) N-laurylsarcosine Sodium salt/ 0.2% (w/v) ascorbic acid/0.2mM DTT/ 2 mM benzamidine/ 2mM PMSF

Table E-2. Cry1Da_7 Extraction Parameter [1]

Sample Type	Target Tissue to Buffer Ratio	Extraction Buffer
OSL1	1:100	100 mM Sodium Carbonate Extraction Solution[2]
OSR1	1:100	100 mM Sodium Carbonate Extraction Solution[2]
OSL4	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Silk	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Pollen	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Forage	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Forage Root	1:100	100 mM Sodium Carbonate Extraction Solution[2]
Grain	1:100	100 mM Sodium Carbonate Extraction Solution[2]

[1] Cry1Da_7 protein was extracted by adding the appropriate volume of extraction buffer, metal beads, and shaking in a Geno Grinder (SPEX Inc., Metuchen, NJ). The extracted samples were clarified by centrifugation

[2] 100 mM Na_2CO_3/ 0.2% (v/v) Tween 20, 0.2% (v/v) N-laurylsarcosine Sodium salt/ 0.2% (w/v) ascorbic acid/0.2mM DTT/ 2 mM benzamidine/ 2mM PMSF

E.5. ELISA Reagents and Methods

E.5.1. Cry1B.868 Antibodies

Mouse anti- Cry1B.868 monoclonal antibody (clone 9E8; lot 658272A) is used as the capture antibody. This antibody was purified using Protein G affinity chromatography and stored in phosphate buffered saline, pH 7.2. The concentration of the purified antibody was determined to be 3.21 mg/mL by spectrophotometric methods.

Mouse anti- Cry1B.868 antibody (clone 3B8; lot 634038) was purified using Protein G affinity chromatography. The purified antibodies were coupled with biotin (Thermo Fisher Scientific), according to the manufacturer's instructions and stored in phosphate buffered saline, pH 7.2.

The detection reagent was NeutrAvidin conjugated to horseradish peroxidase (Thermo Fisher Scientific).

E.5.2. Cry1Da_7 Antibodies

Mouse anti-Cry1Da_7 monoclonal antibody (clone 5231-6; lot 617720) is used as the capture antibody. This antibody was purified using Protein A affinity chromatography and stored in phosphate buffered saline, pH 7.2. The concentration of the purified antibody was determined to be 3.59 mg/mL by spectrophotometric methods.

Protein A affinity chromatography purified monoclonal antibody (mouse anti-Cry1Da_7, (clone 5231-18; lot 617506) was coupled with biotin (Thermo Fisher Scientific), per the manufacturer's instructions and stored in phosphate buffered saline, pH 7.2.

The detection reagent was NeutrAvidin conjugated to horseradish peroxidase (Thermo Fisher Scientific).

E.5.3. Cry1B.868 ELISA Method

The Cry1B.868 ELISA was performed according to the current version of method ME-2206. Mouse anti-Cry1B.868 capture antibody, was diluted in a coating buffer (0.015 M Na2CO3 and 0.035 M $NaHCO_3$ with 150mM NaCl) to 3 µg/ml and immobilized onto microtiter plates followed by incubation in a 4ºC refrigerator for ≥8 h. Prior to each step in the assay, plates were washed with 1X phosphate buffered saline containing 0.05% (v/v) Tween 20. Cry1B.868 protein standard or sample extract diluted in assay buffer (0.1% (w/v) bovine serum albumin in 1X phosphate buffered saline containing 0.05% (v/v) Tween 20) was added incubated for 60 ± 10 minutes at 37 ºC. Biotinylated mouse anti-Cry1B.868 was added and incubated for 60 ± 10 minutes at 37 ºC. NeutrAvidin-horseradish peroxidase conjugate was added and incubated for 60 ± 10 minutes at 37 ºC. Plates were developed by adding horseradish peroxidase substrate, 3,3',5,5'- tetramethylbenzidine (TMB, Sigma-Aldrich). The enzymatic reaction was terminated by the addition of 6 M H_3PO_4. Quantification of Cry1B.868 protein was accomplished by interpolation from a Cry1B.868 protein standard curve that ranged from 0.625 – 20 ng/ml.

E.5.4. Cry1Da_7 ELISA Method

The Cry1Da_7 ELISA was performed per the current version of method ME-2203. The capture antibody, mouse anti-Cry1Da_7 monoclonal antibody, was diluted in a coating buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$ with 150mM NaCl) and immobilized onto a microtiter plate at 4 µg/ml followed by incubation in a 4ºC refrigerator for ≥8 h. Prior to each step in the assay, plates were washed with 1X phosphate buffered saline containing 0.05% (v/v) Tween 20. Cry1Da_7 protein standard or sample extract diluted in assay buffer (0.1% (w/v) bovine serum albumin in 1X phosphate buffered saline containing 0.05% (v/v) Tween 20) was added to the plate and incubated for 60 ± 10 minutes at 37 ºC. Biotinylated mouse anti-Cry1Da_7 monoclonal antibody was added to the plate and incubated for 60 ± 10 minutes at 37 ºC. NeutrAvidin-horseradish peroxidase conjugate was added at to the plate and incubated for 60 ± 10 minutes at 37 ºC. Plates were developed by adding horseradish peroxidase substrate, 3,3',5,5'- tetramethylbenzidine (TMB, Sigma-Aldrich). The enzymatic reaction was terminated by the addition 6 M H_3PO_4. Quantification of Cry1Da_7 protein was accomplished by interpolation from a Cry1Da_7 protein standard curve that ranged from 0.250 – 8 ng/ml.

E.6. Data Analyses

Cry1B.868 and Cry1Da_7 ELISA plates were analyzed on a SPECTRAmax Plus 384 (Molecular Devices) microplate spectrophotometer, using a dual wavelength detection method. Protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GxP software. Absorbance readings and protein standard concentrations were fitted with a five-parameter curve fit.

Following the interpolation from the standard curve, for data that were determined to be greater than or equal to the LOQ, the protein levels (ng/ml) in the tissues were converted to a µg/g dw value. For each protein, this conversion utilized a sample dilution factor and a tissue-to-buffer ratio and an extraction efficiency correction factor. All values were also converted to a µg/g fw using moisture conversion factor (MCF) (Table E-3).

Table E-3. Summary of the Tissue-Specific Moisture Conversion Factors[1]

Tissue Type	N	Average MCF	Standard Deviation
OSL1	56	0.15	0.02
OSR1	60	0.11	0.02
OSL4	31	0.23	0.03
Silk	21	0.10	0.01
Pollen	56	0.57	0.11
Forage	51	0.30	0.04
Forage Root	55	0.19	0.04
Grain	56	0.88	0.02

[1] Universal moisture conversion factor for specific tissues based on historical data for the estimation of fw protein expression levels from protein expression levels reported on µg/g dw basis.

Core LIMS (Core Informatics) and its supporting tools were used to calculate the protein levels in maize tissues. The sample means, standard errors (SEs), and ranges were calculated by R code. All protein expression levels were rounded to two significant figures

Appendix F: Digestive Fate of the Cry1B.868 and Cry1Da_7 Proteins

F.1. Digestive Fate of the Cry1B.868 and Cry1Da_7 Proteins

As part of a comprehensive evaluation, introduced proteins in biotechnology-derived crops are evaluated for their safety for human and animal consumption. Proteins are an essential dietary component for humans and animals, and most are rapidly degraded to the component amino acids for nutritional purposes (Hammond and Jez, 2011). Although the vast majority of ingested proteins are non-allergenic, a small set of proteins or their fragments have been associated with a variety of gastrointestinal and systemic manifestations of immune-mediated allergy. One characteristic of several food allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood et al., 1996; Moreno et al., 2005; Vassilopoulou et al., 2006; Vieths et al., 1999), although exceptions to the correlation can be identified (Fu et al., 2002). The enzymatic degradation of an ingested protein by exposure to gastric pepsin and/or intestinal pancreatic proteases (e.g., pancreatin) makes it highly unlikely that either the intact protein or protein fragment(s) will reach the absorptive epithelial cells of the small intestine where antigen processing cells reside (Moreno et al., 2005). Therefore, the susceptibility of Cry1B.868 and Cry1Da_7 to the presence of pepsin was evaluated using an assay protocol that has been standardized based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). The susceptibility of proteins in the presence of pancreatin has also been used as a separate test system to assess the digestibility of food components (Okunuki et al., 2002; Yagami et al., 2000).

F.1.1. Degradation of Cry1B.868 and Cry1Da_7 Proteins in the Presence of Pepsin

Degradation of the Cry1B.868 and Cry1Da_7 proteins by pepsin was evaluated over time by analyzing digestion mixtures incubated for targeted time intervals following a standardized protocol validated in an international, multi-laboratory ring study (Thomas et al., 2004) collected at targeted incubation time points. The specific methods used to assess the digestive fate/degradation of the Cry1B.868 and Cry1Da_7 proteins in pepsin are summarized below and detailed in Appendix F.2. The study showed that the results of *in vitro* pepsin digestion assays using this protocol were reproducible and consistent for determining the digestive susceptibility of a protein. This standardized *in vitro* pepsin digestion protocol utilized a physiologically relevant acidic buffer appropriate for pepsin activity. The susceptibility of Cry1B.868 and Cry1Da_7 proteins to pepsin degradation was assessed by visual analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel and by visual analysis of a western blot probed with anti-Cry1B.868 or anti-Cry1Da_7 polyclonal antibodies. Both visualization methods were run concurrently with separate SDS-PAGE and western blot analyses to estimate the limit of detection (LOD) of the Cry1B.868 and Cry1Da_7 proteins for each method.

F.1.1.1. Degradation of Cry1B.868 Protein in the Presence of Pepsin

For SDS-PAGE analysis of the digestibility of the Cry1B.868 protein in pepsin, the gel was loaded with 1 µg of total test protein (based on pre-digestion protein concentrations) for each of the digestion samples (Figure F-1, Panel A). The SDS-PAGE gel for the digestibility assessment was run concurrently with a separate SDS-PAGE gel to estimate the LOD of the Cry1B.868 protein (Figure F-1, Panel B). The LOD of intact Cry1B.868 protein was approximately 1.6 ng (Figure F-1, Panel B, lane 9). Visual examination of SDS-PAGE data showed that the intact Cry1B.868 protein was digested within 0.5 min of incubation in pepsin (Figure F-1, Panel A, lane 5). Therefore, based on the LOD, more than 99.8% (100% - 0.2% = 99.8%) of the intact Cry1B.868 protein was digested within 0.5 min of incubation in pepsin. Transiently-stable peptide fragments at ~4-kDa were observed throughout the course of the digestion.

No change in the Cry1B.868 protein band intensity was observed in the absence of pepsin in the 0 min No Pepsin Control and 60 min No Pepsin Control (Figure F-1, Panel A, lanes 3 and 12). This indicates that the degradation of the Cry1B.868 protein was due to the proteolytic activity of pepsin and not due to instability of the protein while incubated in 10 mM HCl, 2 mg/ml NaCl, pH ~1.2 for 60 min.

The 0 min No Test Protein Control and 60 min No Test Protein Control (Figure F-1, Panel A, lanes 2 and 13) demonstrated that the pepsin is stable throughout the experimental phase.

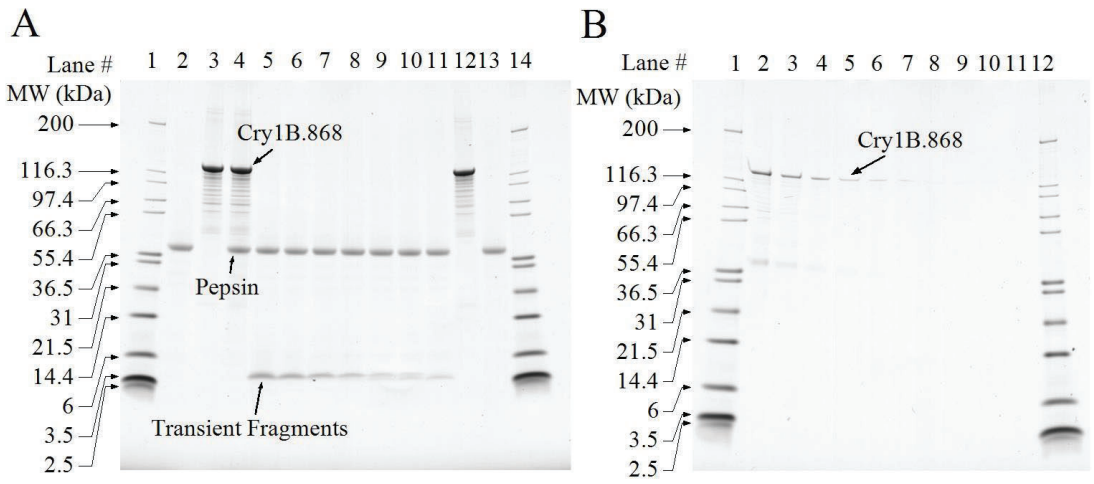

Figure F-1. SDS-PAGE Analysis of the Degradation of Cry1B.868 Protein by Pepsin

Colloidal Brilliant Blue G stained SDS-PAGE gels were used to assess the degradation of Cry1B.868 protein by pepsin. Molecular weights (kDa) are shown on the left of each gel and correspond to the markers loaded. In each gel, the Cry1B.868 protein migrated to approximately 129.6 kDa and pepsin to approximately 38 kDa. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images.

A: Cry1B.868 protein degradation in the presence of pepsin. Based on pre-reaction protein concentrations, 1 µg of test protein was loaded in each lane containing Cry1B.868 protein.

B: LOD determination. Indicated amounts of the test protein from the Pepsin Treated T0 sample were loaded to estimate the LOD of the Cry1B.868 protein.

A

Lane	Sample	Incubation Time (min)
1	Mark12 MWM	-
2	0 min No Test Protein Control	0
3	0 min No Pepsin Control	0
4	Pepsin Treated T0	0
5	Pepsin Treated T1	0.5
6	Pepsin Treated T2	2
7	Pepsin Treated T3	5
8	Pepsin Treated T4	10
9	Pepsin Treated T5	20
10	Pepsin Treated T6	30
11	Pepsin Treated T7	60
12	60 min No Pepsin Control	60
13	60 min No Test Protein Control	60
14	Mark12 MWM	-
15	Empty	-

B

Lane	Sample	Amount (ng)
1	Mark12 MWM	-
2	Pepsin Treated T0	200
3	Pepsin Treated T0	100
4	Pepsin Treated T0	50
5	Pepsin Treated T0	25
6	Pepsin Treated T0	12.5
7	Pepsin Treated T0	6.25
8	Pepsin Treated T0	3.1
9	Pepsin Treated T0	1.6
10	Pepsin Treated T0	0.8
11	Pepsin Treated T0	0.4
12	Mark12 MWM	-
13	Empty	-
14	Empty	-
15	Empty	-

For western blot analysis of Cry1B.868 pepsin susceptibility, the Cry1B.868 protein was loaded with approximately 40 ng per lane of total protein (based on pre-reaction total protein concentrations) for each reaction time point examined. The western blot used to assess Cry1B.868 protein degradation (Figure F-2, Panel A) was run concurrently with the western blot used to

estimate the LOD (Figure F-2, Panel B). The LOD of the Cry1B.868 protein was approximately 0.63 ng (Figure F-2, Panel B, Lane 9). Western blot analysis demonstrated that the intact Cry1B.868 protein was degraded below the LOD within 0.5 min of incubation in the presence of pepsin (Figure F-2, Panel A, Lane 6). Based on the western blot LOD for the Cry1B.868 protein, more than 98.4% (100% - 1.6% = 98.4%) of the intact Cry1B.868 protein was degraded within 0.5 min. No peptide fragments were detected at the 0.5 min and beyond time points in the western blot analysis.

No change in the Cry1B.868 protein band intensity was observed in the absence of pepsin in the 0 min No Pepsin Control and 60 min No Pepsin Control (Figure F-2, Panel A, lanes 3 and 12). This indicates that the degradation of the Cry1B.868 protein was due to the proteolytic activity of pepsin and not due to instability of the protein while incubated in 2 mg/ml NaCl, 10 mM HCl, pH ~1.2 for 60 min. The transiently-stable fragments at ~4 kDa that were observed by SDS-PAGE were not recognized by the antibody used in this western blot.

No immunoreactive bands were observed in 0 min No Protein Control and 60 min No Protein Control (Figure F-2, Panel A, lanes 3 and 14). This result indicates that there was no non-specific interaction between the pepsin solution and the Cry1B.868-specific antibody under these experimental conditions.

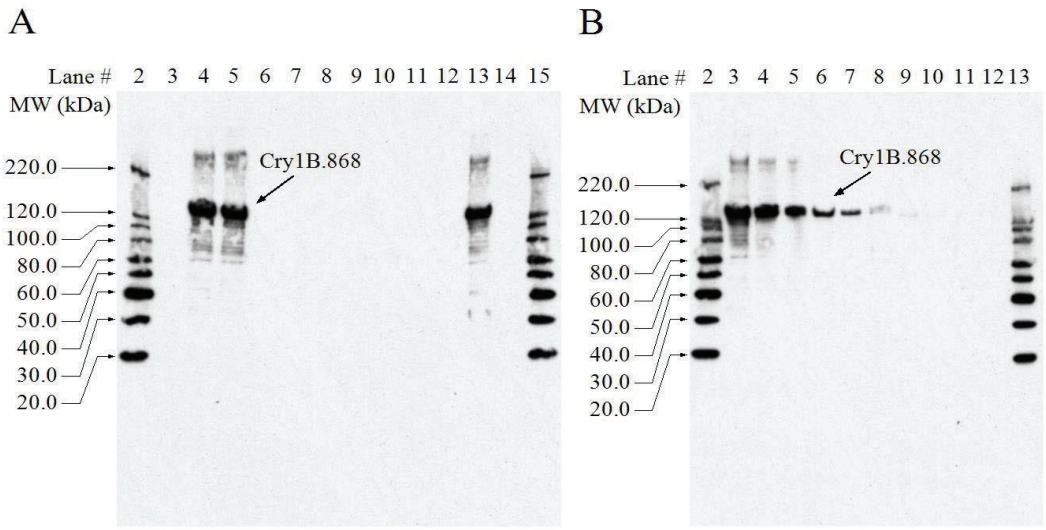

Figure F-2. Western Blot Analysis of the Degradation of Cry1B.868 Protein by Pepsin

Western blots probed with an anti-Cry1B.868 antibody were used to assess the degradation of Cry1B.868 by pepsin. Molecular weights (kDa) are shown on the left of each gel and correspond to the MagicMark™ molecular weight marker. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images. A 1.5 min-exposure is shown.
A: Cry1B.868 protein degradation by pepsin. Based on pre-reaction protein concentrations, 40 ng of test protein was loaded in each lane containing Cry1B.868 protein.
B: LOD determination. Indicated amounts of the test protein from the Pepsin Treated T0 sample were loaded to estimate the LOD of the Cry1B.868 protein.

A

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	0 min No Test Protein Control	0
4	0 min No Pepsin Control	0
5	Pepsin Treated T0	0
6	Pepsin Treated T1	0.5
7	Pepsin Treated T2	2
8	Pepsin Treated T3	5
9	Pepsin Treated T4	10
10	Pepsin Treated T5	20
11	Pepsin Treated T6	30
12	Pepsin Treated T7	60
13	60 min No Pepsin Control	60
14	60 min No Test Protein Control	60
15	MagicMark MWM	-

B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	Pepsin Treated T0	40
4	Pepsin Treated T0	20
5	Pepsin Treated T0	10
6	Pepsin Treated T0	5
7	Pepsin Treated T0	2.5
8	Pepsin Treated T0	1.25
9	Pepsin Treated T0	0.63
10	Pepsin Treated T0	0.31
11	Pepsin Treated T0	0.16
12	Pepsin Treated T0	0.08
13	MagicMark MWM	-
14	Precision Plus MWM	-
15	Empty	-

F.1.1.2. Degradation of Cry1Da_7 Protein in the Presence of Pepsin

For SDS-PAGE analysis of the digestibility of the Cry1Da_7 protein in pepsin, the gel was loaded with 1 µg of total test protein (based on pre-digestion protein concentrations) for each of the digestion samples (Figure F-3, Panel A). The SDS-PAGE gel for the digestibility assessment was run concurrently with a separate SDS-PAGE gel to estimate the LOD of the Cry1Da_7 protein (Figure F-3, Panel B). The LOD of intact Cry1Da_7 protein was approximately 6.3 ng (Figure F-3, Panel B, lane 8). Visual examination of SDS-PAGE data showed that the intact Cry1Da_7 protein was digested within 0.5 min of incubation in pepsin (Figure F-3, Panel A, lane 5). Therefore, based on the LOD, more than 99.4% (100% - 0.6% = 99.4%) of the intact Cry1Da_7 protein was digested within 0.5 min of incubation in pepsin. Transiently-stable peptide fragments at ~4-kDa were observed throughout the course of the digestion.

No change in the Cry1Da_7 protein band intensity was observed in the absence of pepsin in the 0 min No Pepsin Control and 60 min No Pepsin Control (Figure F-3, Panel A, lanes 3 and 12). This indicates that the degradation of the Cry1Da_7 protein was due to the proteolytic activity of pepsin and not due to instability of the protein while incubated in 10 mM HCl, 2 mg/ml NaCl, pH ~1.2 for 60 min.

The 0 min No Test Protein Control and 60 min No Test Protein Control (Figure F-3, Panel A, lanes 2 and 13) demonstrated that the pepsin is stable throughout the experimental phase.

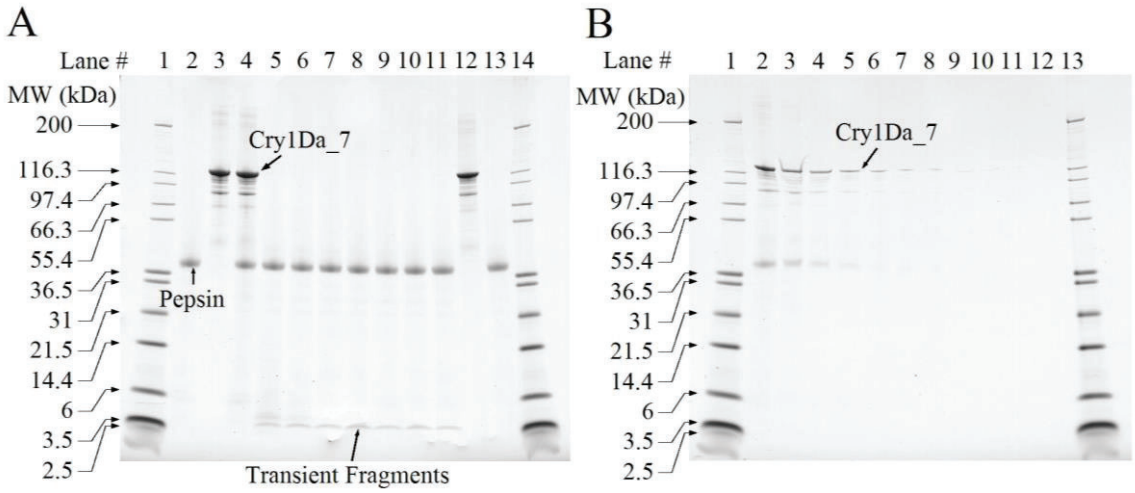

Figure F-3. SDS-PAGE Analysis of the Degradation of Cry1Da_7 Protein by Pepsin

Colloidal Brilliant Blue G stained SDS-PAGE gels were used to assess the degradation of Cry1Da_7 protein by pepsin. Molecular weights (kDa) are shown on the left of each gel and correspond to the markers loaded. In each gel, the Cry1Da_7 protein migrated to approximately 126.5 kDa and pepsin to approximately 38 kDa. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images.

A: Cry1Da_7 protein degradation in the presence of pepsin. Based on pre-reaction protein concentrations, 1 µg of test protein was loaded in each lane containing Cry1Da_7 protein.

B: LOD determination. Indicated amounts of the test protein from the Pepsin Treated T0 sample were loaded to estimate the LOD of the Cry1Da_7 protein.

A

Lane	Sample	Incubation Time (min)
1	Mark12 MWM	-
2	0 min No Test Protein Control	0
3	0 min No Pepsin Control	0
4	Pepsin Treated T0	0
5	Pepsin Treated T1	0.5
6	Pepsin Treated T2	2
7	Pepsin Treated T3	5
8	Pepsin Treated T4	10
9	Pepsin Treated T5	20
10	Pepsin Treated T6	30
11	Pepsin Treated T7	60
12	60 min No Pepsin Control	60
13	60 min No Test Protein Control	60
14	Mark12 MWM	-
15	Empty	-

B

Lane	Sample	Amount (ng)
1	Mark12 MWM	-
2	Pepsin Treated T0	400
3	Pepsin Treated T0	200
4	Pepsin Treated T0	100
5	Pepsin Treated T0	50
6	Pepsin Treated T0	25
7	Pepsin Treated T0	12.5
8	Pepsin Treated T0	6.3
9	Pepsin Treated T0	3.1
10	Pepsin Treated T0	1.6
11	Pepsin Treated T0	0.8
12	Pepsin Treated T0	0.4
13	Mark12 MWM	-
14	Empty	-
15	Empty	-

For western blot analysis of Cry1Da_7 pepsin susceptibility, the Cry1Da_7 protein was loaded with approximately 40 ng per lane of total protein (based on pre-reaction total protein concentrations) for each reaction time point examined. The western blot used to assess Cry1Da_7 protein degradation (Figure F-4, Panel A) was run concurrently with the western blot used to estimate the LOD (Figure F-4, Panel B). The LOD of the Cry1Da_7 protein was approximately 1.25 ng (Figure F-4, Panel B, Lane 7). Western blot analysis demonstrated that the intact Cry1Da_7 protein was degraded below the LOD within 0.5 min of incubation in the presence of pepsin (Figure F-4, Panel A, Lane 6). Based on the western blot LOD for the Cry1Da_7 protein, more than 96.9% (100% - 3.1% = 96.9%) of the intact Cry1Da_7 protein was degraded within 0.5 min. No peptide fragments were detected at the 0.5 min and beyond time points in the western blot analysis.

No change in the Cry1Da_7 protein band intensity was observed in the absence of pepsin in the 0 min No Pepsin Control and 60 min No Pepsin Control (Figure F-4, Panel A, lanes 3 and 12). This indicates that the degradation of the Cry1Da_7 protein was due to the proteolytic activity of pepsin and not due to instability of the protein while incubated in 2 mg/ml NaCl, 10 mM HCl, pH ~1.2 for 60 min. The transiently-stable fragments at ~4 kDa that were observed by SDS-PAGE were not recognized by the antibody used in this western blot.

No immunoreactive bands were observed in 0 min No Protein Control and 60 min No Protein Control (Figure F-4, Panel A, lanes 3 and 14). This result indicates that there was no non-specific interaction between the pepsin solution and the Cry1Da_7-specific antibody under these experimental conditions.

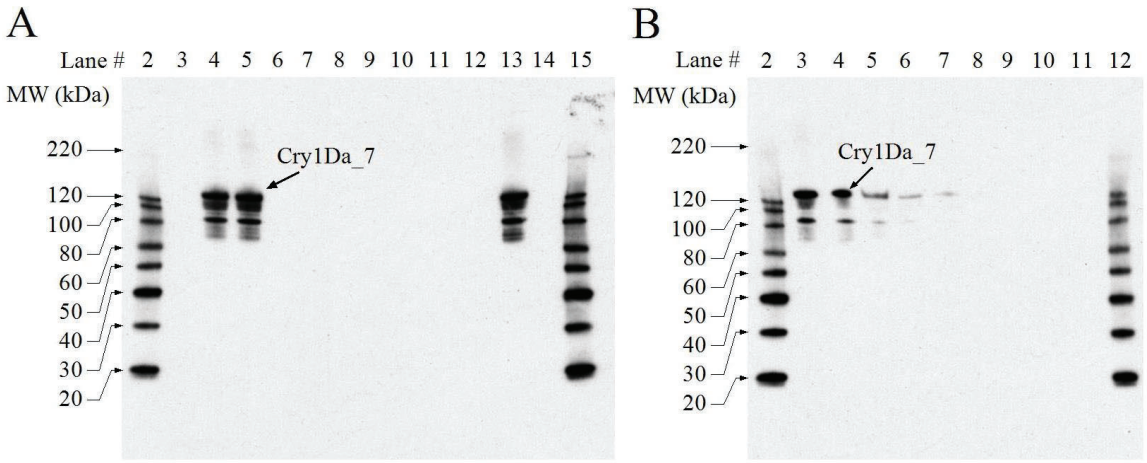

Figure F-4. Western Blot Analysis of the Degradation of Cry1Da_7 Protein by Pepsin

Western blots probed with an anti-Cry1Da_7 antibody were used to assess the degradation of Cry1Da_7 by pepsin. Molecular weights (kDa) are shown on the left of each gel and correspond to the MagicMark™ molecular weight marker. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images. A 1.5 min exposure is shown.

A: Cry1Da_7 protein degradation by pepsin. Based on pre-reaction protein concentrations, 40 ng of test protein was loaded in each lane containing Cry1Da_7 protein.

B: LOD determination. Indicated amounts of the test protein from the Pepsin Treated T0 sample were loaded to estimate the LOD of the Cry1Da_7 protein.

A

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	0 min No Test Protein Control	0
4	0 min No Pepsin Control	0
5	Pepsin Treated T0	0
6	Pepsin Treated T1	0.5
7	Pepsin Treated T2	2
8	Pepsin Treated T3	5
9	Pepsin Treated T4	10
10	Pepsin Treated T5	20
11	Pepsin Treated T6	30
12	Pepsin Treated T7	60
13	60 min No Pepsin Control	60
14	60 min No Test Protein Control	60
15	MagicMark MWM	-

B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	Pepsin Treated T0	20
4	Pepsin Treated T0	10
5	Pepsin Treated T0	5
6	Pepsin Treated T0	2.5
7	Pepsin Treated T0	1.25
8	Pepsin Treated T0	0.63
9	Pepsin Treated T0	0.31
10	Pepsin Treated T0	0.16
11	Pepsin Treated T0	0.08
12	MagicMark MWM	-
13	Precision Plus MWM	-
14	Empty	-
15	Empty	-

F.1.2. Degradation of Cry1B.868 and Cry1Da_7 Proteins in the Presence of Pancreatin

F.1.2.1. Degradation of Cry1B.868 Protein in the Presence of Pancreatin

The degradation of the Cry1B.868 protein by pancreatin was assessed by western blot analysis (Figure F-5). The western blot used to assess the Cry1B.868 protein degradation (Figure F-5, Panel A) was run concurrently with the western blot used to estimate the LOD (Figure F-5, Panel B) of the Cry1B.868 protein. The LOD of the Cry1B.868 protein was observed at approximate 0.16 ng protein loading (Figure F-5, Panel B, lane 11). The LOD was used to calculate the maximum relative amount of Cry1B.868 protein that could remain visually undetected after digestion, which corresponded to approximately 0.4% of the total protein loaded.

The Cry1B.868 protein was loaded with approximately 40 ng per lane of total protein (based on pre-reaction total protein concentrations) for each reaction time point examined. Western blot analysis demonstrated that the intact Cry1B.868 protein was degraded below the LOD within 5 min of incubation in the presence of pancreatin (Figure F-5, Panel A, lane 5). Based on the western blot LOD for the Cry1B.868 protein, more than 99.6% (100% - 0.4% = 99.6%) of the intact Cry1B.868 protein was degraded within 5 min. Bands of ~60 kDa corresponding to fragments of Cry1B.868 were present over the course of the assessment (Figure F-5, Panel A, lanes 5-12). The Cry1B.868 demonstrated typical characteristics that are present in many other Cry proteins, such as Cry1A.105 (Wang et al 2018, USDA-APHIS Petition #13-337-01p p75) and Cry2Ab2 (USDA-APHIS Petition #13-337-01p p75).

No obvious change in the intact Cry1B.868 (~129.6 kDa) band intensity was observed in the absence of pancreatin in the 0 min No Pancreatin Control and 24 hour No Pancreatin Control (Figure F-5, Panel A, lanes 3 and 13). This indicates that the degradation of all immunoreactive forms of the Cry1B.868 protein was due to the proteolytic activity of pancreatin and not due to instability of the protein when incubated in 50 mM KH_2PO_4, pH 7.5 over the course of the experiment.

No immunoreactive bands were observed in the 0 min No Test Protein Control and 24 hour No Test Protein Control (Figure F-5, Panel A, lanes 2 and 14), demonstrating the absence of non-specific antibody interactions with the pancreatin solution.

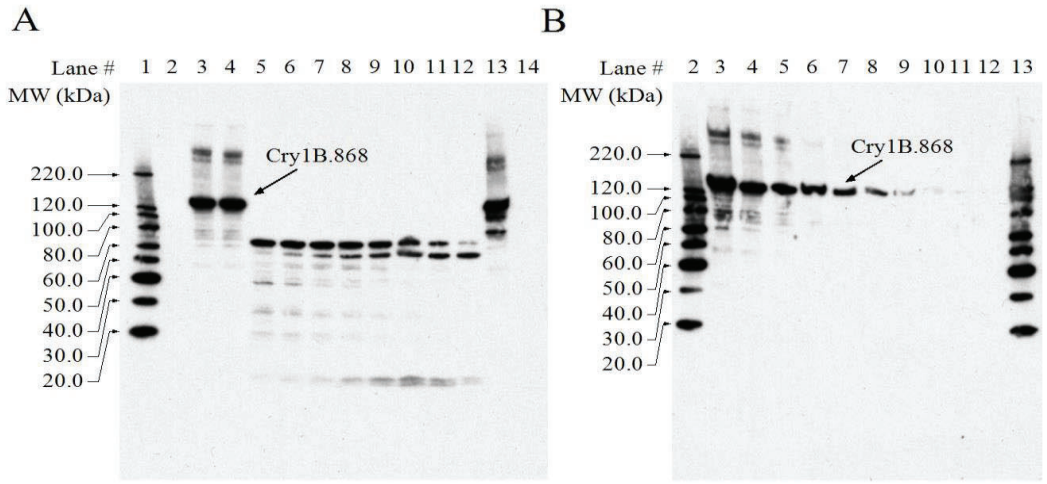

Figure F-5. Western Blot Analysis of the Degradation of Cry1B.868 Protein by Pancreatin

Western blots probed with an anti-Cry1B.868 antibody were used to assess the degradation of Cry1B.868 by pancreatin. Molecular weights (kDa) are shown on the left of each gel and correspond to the MagicMark™ molecular weight marker. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images. A 1.0 min-exposure is shown.

A: Cry1B.868 protein degradation by pancreatin. Based on pre-reaction protein concentrations, 40 ng of test protein was loaded in each lane containing Cry1B.868 protein.

B: LOD determination. Indicated amounts of the test protein from the Pancreatin Treated T0 sample were loaded to estimate the LOD of the Cry1B.868 protein.

A

Lane	Sample	Incubation Time
1	MagicMark MWM	-
2	0 min No Test Protein Control	0
3	0 min No Pancreatin Control	0
4	Pancreatin Treated T0	0
5	Pancreatin Treated T1	5 min
6	Pancreatin Treated T2	15 min
7	Pancreatin Treated T3	30 min
8	Pancreatin Treated T4	1 h
9	Pancreatin Treated T5	2 h
10	Pancreatin Treated T6	4 h
11	Pancreatin Treated T7	8 h
12	Pancreatin Treated T8	24 h
13	24 h No Pancreatin Control	24 h
14	24 h No Test Protein Control	24 h
15	Precision Plus MWM	-

B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	Pancreatin Treated T0	40
4	Pancreatin Treated T0	20
5	Pancreatin Treated T0	10
6	Pancreatin Treated T0	5
7	Pancreatin Treated T0	2.5
8	Pancreatin Treated T0	1.25
9	Pancreatin Treated T0	0.63
10	Pancreatin Treated T0	0.31
11	Pancreatin Treated T0	0.16
12	Pancreatin Treated T0	0.08
13	MagicMark MWM	-
14	Precision Plus MWM	-
15	Empty	-

F.1.2.2. Degradation of Cry1Da_7 Protein in the Presence of Pancreatin

The degradation of the Cry1Da_7 protein by pancreatin was assessed by western blot analysis (Figure F-6). The western blot used to assess the Cry1Da_7 protein degradation (Figure F-6, Panel A) was run concurrently with the western blot used to estimate the LOD (Figure F-6, Panel B) of the Cry1Da_7 protein. The LOD of the Cry1Da_7 protein was observed at approximate 1.25 ng protein loading (Figure F-6, Panel B, lane 7). The LOD was used to calculate the maximum relative amount of Cry1Da_7 protein that could remain visually undetected after digestion, which corresponded to approximately 3.1% of the total protein loaded.

The Cry1Da_7 protein was loaded with approximately 40 ng per lane of total protein (based on pre-reaction total protein concentrations) for each reaction time point examined. Western blot analysis demonstrated that the intact Cry1Da_7 protein was degraded below the LOD within 5 min of incubation in the presence of pancreatin (Figure F-6, Panel A, lane 5). Based on the western blot LOD for the Cry1Da_7 protein, more than 96.9% (100% - 3.1% = 96.9%) of the intact Cry1Da_7 protein was degraded within 5 min. A band of ~60 kDa corresponding to a fragment of Cry1Da_7 was present over the course of the assessment (Figure F-6, Panel A, lanes 5-12). The Cry1Da_7 demonstrated typical characteristics that are present in many other Cry proteins, such as Cry1A.105 (Wang et al 2018, USDA-APHIS Petition #13-337-01p p75) and Cry2Ab2 (USDA-APHIS Petition #13-337-01p p75).

No obvious change in the intact Cry1Da_7 (~126.5 kDa) band intensity was observed in the absence of pancreatin in the 0 min No Pancreatin Control and 24 hour No Pancreatin Control (Figure F-6, Panel A, lanes 3 and 13). This indicates that the degradation of all immunoreactive forms of the Cry1Da_7 protein was due to the proteolytic activity of pancreatin and not due to instability of the protein when incubated in 50 mM KH_2PO_4, pH 7.5 over the course of the experiment.

No immunoreactive bands were observed in the 0 min No Test Protein Control and 24 hour No Test Protein Control (Figure F-6, Panel A, lanes 2 and 14), demonstrating the absence of non-specific antibody interactions with the pancreatin solution.

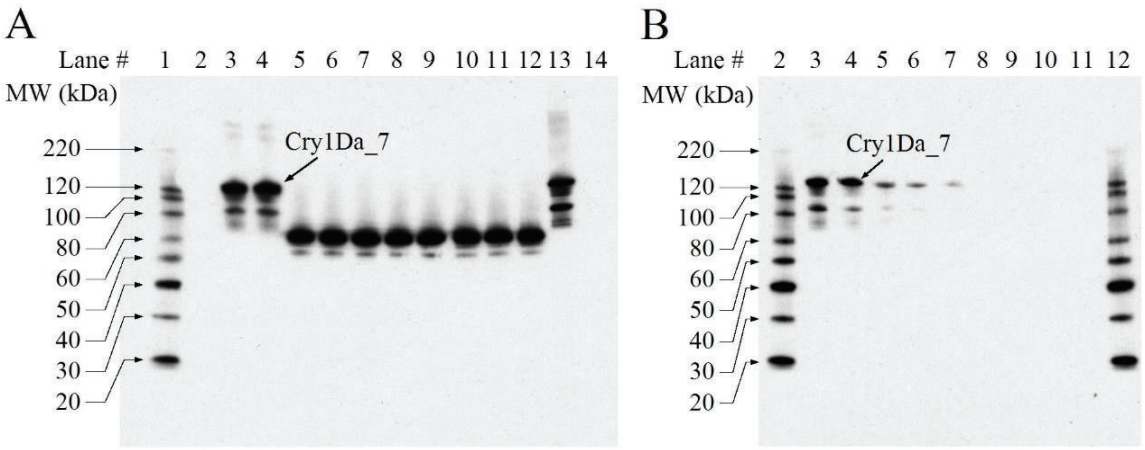

Figure F-6. Western Blot Analysis of the Degradation of Cry1Da_7 Protein by Pancreatin

Western blots probed with an anti-Cry1Da_7 antibody were used to assess the degradation of Cry1Da_7 by pancreatin. Molecular weights (kDa) are shown on the left of each gel and correspond to the MagicMark™ molecular weight marker. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images. A 1.5 min exposure is shown.

A: Cry1Da_7 protein degradation by pancreatin. Based on pre-reaction protein concentrations, 40 ng of test protein was loaded in each lane containing Cry1Da_7 protein.

B: LOD determination. Indicated amounts of the test protein from the Pancreatin Treated T0 sample were loaded to estimate the LOD of the Cry1Da_7 protein.

A

Lane	Sample	Incubation Time
1	MagicMark MWM	-
2	0 min No Test Protein Control	0
3	0 min No Pancreatin Control	0
4	Pancreatin Treated T0	0
5	Pancreatin Treated T1	5 min
6	Pancreatin Treated T2	15 min
7	Pancreatin Treated T3	30 min
8	Pancreatin Treated T4	1 h
9	Pancreatin Treated T5	2 h
10	Pancreatin Treated T6	4 h
11	Pancreatin Treated T7	8 h
12	Pancreatin Treated T8	24 h
13	24 h No Pancreatin Control	24 h
14	24 h No Test Protein Control	24 h
15	Precision Plus MWM	-

B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	Pancreatin Treated T0	20
4	Pancreatin Treated T0	10
5	Pancreatin Treated T0	5
6	Pancreatin Treated T0	2.5
7	Pancreatin Treated T0	1.25
8	Pancreatin Treated T0	0.63
9	Pancreatin Treated T0	0.31
10	Pancreatin Treated T0	0.16
11	Pancreatin Treated T0	0.08
12	MagicMark MWM	-
13	Precision Plus MWM	-
14	Empty	-
15	Empty	-

F.1.3. Degradation of Cry1B.868 and Cry1Da_7 Proteins by Pepsin Followed by Pancreatin

To better understand the fate of the transiently-stable peptide fragments at ~4 kDa that were observed in the reaction mixtures throughout the course of the pepsin digestion of Cry1B.868 and Cry1Da_7, sequential digestibility of the Cry1B.868 and Cry1Da_7 proteins were conducted. This sequential digestibility was assessed both by visual analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel, and visual analysis of a western blot probed with an anti Cry1B.868-polyclonal and Cry1Da_7-polyclonal antibodies.

F.1.3.1. Degradation of Cry1B.868 Protein by Pepsin Followed by Pancreatin

For the sequential degradation assay, the Cry1B.868 protein was incubated with pepsin for 2 min, followed by incubation with pancreatin. For the Colloidal Brilliant Blue G stained SDS-PAGE assessment, the gel was loaded with 1 µg of Cry1B.868 protein (based on pre-digestion protein concentrations) for each of the digestion samples. Examination of SDS-PAGE data showed that the intact Cry1B.868 protein was digested within 2 min of incubation in pepsin (Figure F-7, Panel A, lane 3) and the small transient fragments at ~4 kDa was completely digested within 0.5 min of pancreatin exposure (Figure F-7, Panel A, lane 7).

No change in the fragment band intensities was observed in the absence of pancreatin in the SEQ 0 min No Pancreatin Control and SEQ 2 hour No Pancreatin Control (Figure F-7, Panel A, lanes 5 and 14). This indicates that the digestion of the fragments was due to the proteolytic activity of pancreatin and not due to instability of the fragment when incubated in 50 mM KH_2PO_4 at pH 7.5 over the course of the experiment.

The SEQ 0 min No Test Protein Control and SEQ 2 hour No Test Protein Control (Figure F-7, Panel A, lanes 4 and 15) demonstrated the integrity of the pancreatin over the course of the experiment. The intensity of some pancreatin bands decreased during the course of the experiment, most likely due to auto-digestion. This is not expected to adversely impact the pancreatin degradation results, as the transiently stable fragments were digested within 0.5 min of exposure to pancreatin.

The sequential digestion of the Cry1B.868 protein was also assessed by western blot (Figure F-7, Panel B), with 40 ng of the test protein (based on total protein pre-digestion concentrations) loaded per lane. No bands were detected in the 2 min Pepsin Treated sample (Figure F-7, Panel B, lane 3).

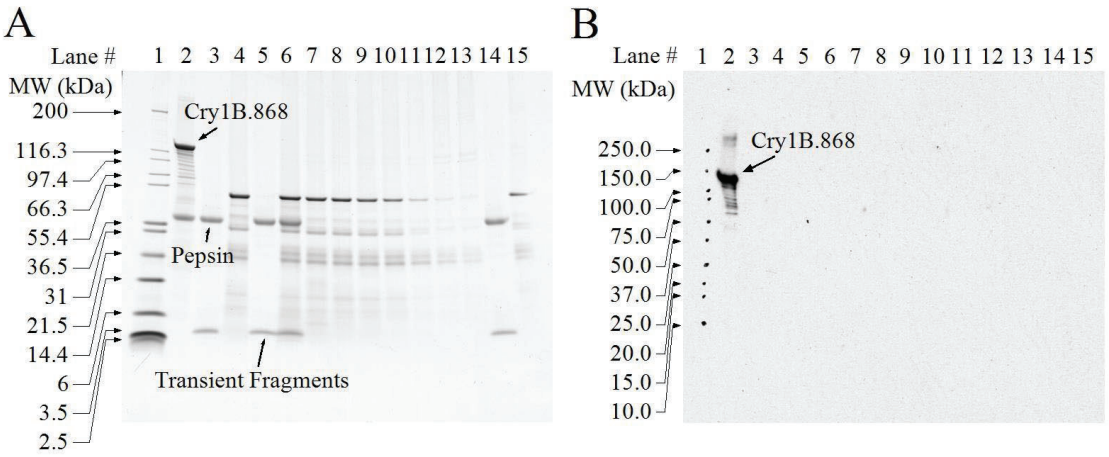

Figure F-7. SDS-PAGE and Western Blot Analysis of the Degradation of Cry1B.868 Protein by Sequential Digestion

SDS-PAGE and western blot analysis were used to assess the degradation of Cry1B.868 in sequential digestion. Molecular weights (kDa) are shown on the left of each gel and correspond to the markers loaded.
A: Colloidal Brilliant Blue G stained SDS-PAGE gel analysis of Cry1B.868 in sequential digestion. Based on pre-digestion protein concentrations, 1 µg of test protein was loaded in each lane containing Cry1B.868 protein.
B: Western blot analysis of Cry1B.868 in sequential digestion. Based on pre-digestion protein concentrations, 40 ng of test protein was loaded in each lane containing Cry1B.868 protein. A 1.0 min-exposure is shown.

A

Lane	Sample	Incubation Time
1	Mark12 MWM	-
	Pepsin Degradation	
2	0 min Pepsin Treated	0 min
3	2 min Pepsin Treated	2 min
	Pancreatin Degradation	
4	SEQ 0 min No Test Protein Control	0 min
5	SEQ 0 min No Pancreatin Control	0 min
6	SEQ T0	0 min
7	SEQ T1	0.5 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ 2 h No Pancreatin Control	2 h
15	SEQ 2 h No Test Protein Control	2 h

B

Lane	Sample	Incubation Time
1	Precision Plus MWM	-
	Pepsin Degradation	
2	0 min Pepsin Treated	0 min
3	2 min Pepsin Treated	2 min
	Pancreatin Degradation	
4	SEQ 0 min No Test Protein Control	0 min
5	SEQ 0 min No Pancreatin Control	0 min
6	SEQ T0	0 min
7	SEQ T1	0.5 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ 2 h No Pancreatin Control	2 h
15	SEQ 2 h No Test Protein Control	2 h

F.1.3.2. Degradation of Cry1Da_7 Protein by Pepsin Followed by Pancreatin

For the sequential degradation assay, the Cry1Da_7 protein was incubated with pepsin for 2 min, followed by incubation with pancreatin. For the Colloidal Brilliant Blue G stained SDS-PAGE assessment, the gel was loaded with 1 µg of Cry1Da_7 protein (based on pre-digestion protein concentrations) for each of the digestion samples. Examination of SDS-PAGE data showed that the intact Cry1Da_7 protein was digested within 2 min of incubation in pepsin (Figure F-8, Panel A, lane 3) and the small transient fragments at ~4 kDa was completely digested within 0.5 min of pancreatin exposure (Figure F-8, Panel A, lane 7).

No change in the fragment band intensities was observed in the absence of pancreatin in the SEQ 0 min No Pancreatin Control and SEQ 2 hour No Pancreatin Control (Figure F-8, Panel A, lanes 5 and 14). This indicates that the digestion of the fragments was due to the proteolytic activity of pancreatin and not due to instability of the fragment when incubated in 50 mM KH_2PO_4 at pH 7.5 over the course of the experiment.

The SEQ 0 min No Test Protein Control and SEQ 2 hour No Test Protein Control (Figure F-8, Panel A, lanes 4 and 15) demonstrated the integrity of the pancreatin over the course of the experiment. The intensity of some pancreatin bands decreased during the course of the experiment, most likely due to auto-digestion. This is not expected to adversely impact the pancreatin degradation results, as the transiently stable fragments were digested within 0.5 min of exposure to pancreatin.

The sequential digestion of the Cry1Da_7 protein was also assessed by western blot (Figure F-8), with 40 ng of the test protein (based on total protein pre-digestion concentrations) loaded per lane. No bands were detected in the 2 min Pepsin Treated sample (Figure F-8, Panel B, lane 3).

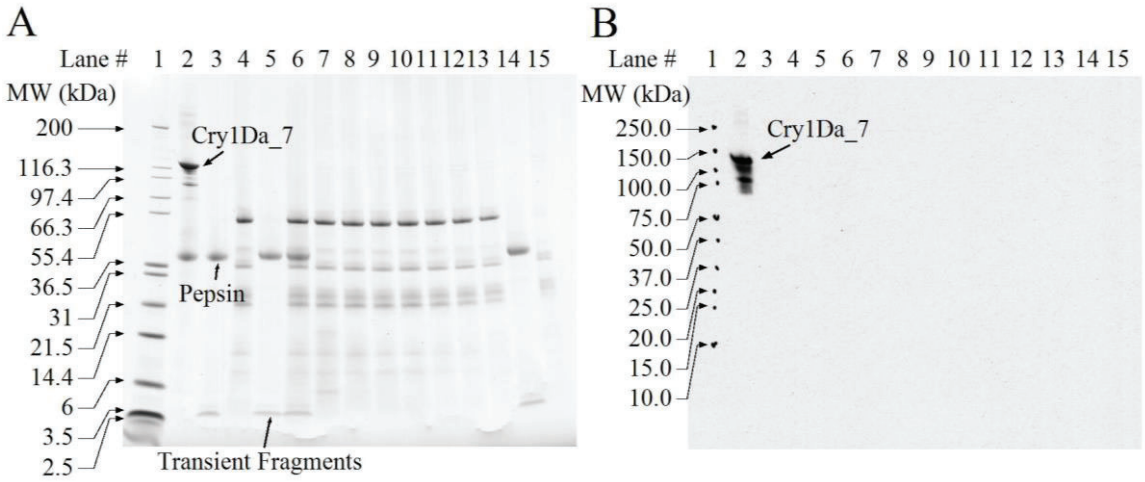

Figure F-8. SDS-PAGE and Western Blot Analysis of the Degradation of Cry1Da_7 Protein by Sequential Digestion

SDS-PAGE and western blot analysis were used to assess the degradation of Cry1Da_7 in sequential digestion. Molecular weights (kDa) are shown on the left of each gel and correspond to the markers loaded.
A: Colloidal Brilliant Blue G stained SDS-PAGE gel analysis of Cry1Da_7 in sequential digestion. Based on pre-digestion protein concentrations, 1 µg of test protein was loaded in each lane containing Cry1Da_7 protein.
B: Western blot analysis of Cry1Da_7 in sequential digestion. Based on pre-digestion protein concentrations, 40 ng of test protein was loaded in each lane containing Cry1Da_7 protein. A 1.0 min exposure is shown.

A

Lane	Sample	Incubation Time
1	Mark12 MWM	-
	Pepsin Degradation	
2	0 min Pepsin Treated	0 min
3	2 min Pepsin Treated	2 min
	Pancreatin Degradation	
4	SEQ 0 min No Test Protein Control	0 min
5	SEQ 0 min No Pancreatin Control	0 min
6	SEQ T0	0 min
7	SEQ T1	0.5 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ 2 h No Pancreatin Control	2 h
15	SEQ 2 h No Test Protein Control	2 h

B

Lane	Sample	Incubation Time
1	Precision Plus MWM	-
	Pepsin Degradation	
2	0 min Pepsin Treated	0 min
3	2 min Pepsin Treated	2 min
	Pancreatin Degradation	
4	SEQ 0 min No Test Protein Control	0 min
5	SEQ 0 min No Pancreatin Control	0 min
6	SEQ T0	0 min
7	SEQ T1	0.5 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ 2 h No Pancreatin Control	2 h
15	SEQ 2 h No Test Protein Control	2 h

F.1.4. Degradation of Cry1B.868 and Cry1Da_7 Proteins Conclusion

F.1.4.1. Degradation of Cry1B.868 Protein Conclusion

The ability of Cry1B.868 protein to be degraded by pepsin and by pancreatin was evaluated in this study. The results showed that at least 99.8% of the intact Cry1B.868 protein was degraded by pepsin within 0.5 min when analyzed by SDS-PAGE and 98.4% of the intact Cry1B.868 was degraded by pepsin within 0.5 min when analyzed by western blot using a Cry1B.868-specific antibody. SDS-PAGE analysis showed that transient peptide fragments at ~4 kDa were observed throughout the course of the pepsin digestion. At least 99.6% of the intact Cry1B.868 protein was degraded by pancreatin within 5 min when analyzed by western blot. These results show that the full-length Cry1B.868 is rapidly degraded by pepsin and pancreatin. The transient fragments at ~4 kDa were rapidly degraded by sequential digestion. Rapid degradation of the intact Cry1B.868 protein by pepsin or pancreatin alone and complete degradation by pepsin followed by pancreatin indicates that the Cry1B.868 protein is highly unlikely to pose any safety concern to human or animal health.

F.1.4.2. Degradation of Cry1Da_7 Protein Conclusion

The ability of Cry1Da_7 protein to be degraded by pepsin and by pancreatin was evaluated in this study. The results showed that at least 99.4% of the intact Cry1Da_7 protein was degraded by pepsin within 0.5 min when analyzed by SDS-PAGE and 96.9% of the intact Cry1Da_7 was degraded by pepsin within 0.5 min when analyzed by western blot using a Cry1Da_7-specific antibody. SDS-PAGE analysis showed that transient peptide fragments at ~4 kDa were observed throughout the course of the pepsin digestion. At least 96.9% of the intact Cry1Da_7 protein was degraded by pancreatin within 5 min when analyzed by western blot. These results show that the full-length Cry1Da_7 is rapidly degraded by pepsin and pancreatin. The transient fragments at ~4 kDa were rapidly degraded by sequential digestion. Rapid degradation of the intact Cry1Da_7 protein by pepsin alone and pancreatin alone and complete degradation by pepsin followed by pancreatin indicates that the Cry1Da_7 protein is highly unlikely to pose any safety concern to human or animal health.

F.2. Materials and Methods Used in Assessing Stability of Cry1B.868 and Cry1Da_7 Proteins Proteins in Pepsin and Pancreatin

F.2.1. Test Substances

F.2.1.1. Cry1B.868 Test Substance

The test substance was the Cry1B.868 protein (lot # 7565, also known as 11479349) purified from *Bacillus thuringiensis* (*Bt*) transformed with the pMON236906 plasmid (*Bt*-produced Cry1B.868, referred to in this document as Cry1B.868 protein). The Cry1B.868 protein is stored in a -80 °C freezer in a buffer solution containing 20 mM CAPS, pH 11.5, 10 mM DTT, 240 mM NaCl, 1 mM Benzamidine (storage buffer).

F.2.1.2. Cry1Da_7 Test Substance

The test substance was the Cry1Da_7 protein (lot # 7799, also known as 11479575) purified from *Bacillus thuringiensis* (*Bt*) transformed with the pMON417133 plasmid (*Bt*-produced Cry1Da_7, referred to in this document as Cry1Da_7 protein). The Cry1Da_7 protein is stored in a -80 °C freezer in a buffer solution containing 25 mM sodium carbonate, pH 10.5 (storage buffer).

F.2.2. Characterization of Test Substance

F.2.2.1. Characterization of the Cry1B.868 Test Substance

The characterization of the test substance was performed under characterization study CHR-2018-0276 at Bayer. The test protein (lot 7565) had a purity of 91%, a total protein concentration of 2.8 mg/ml, and an apparent molecular weight of 129.6 kDa.

F.2.2.2. Characterization of the Cry1Da_7 Test Substance

The characterization of the test substance was performed under characterization study CHR-2018-0365 at Bayer. The test protein (lot 7799) had a purity of 92%, a total protein concentration of 3.6 mg/ml, and an apparent molecular weight of 126.5 kDa.

F.3. Experimental Design

Assays designed to assess the degradation of Cry1B.868 and Cry1Da_7 proteins by pepsin and by pancreatin were performed independently. Because protein fragments attributable to the Cry1B.868 and Cry1Da_7 proteins were identified after 10 min of pepsin degradation, a sequential digestion of the Cry1B.868 and Cry1Da_7 proteins with pepsin followed by pancreatin were also performed.

F.3.1. Preparation of the Pepsin Stock Solution

High purity pepsin (Sigma catalog number P6887; specific activity of 3546 U/mg, 96% protein, purity-corrected specific activity of 3404 U/mg) was dissolved in 10 mM HCl, 2 mg/ml NaCl, pH ~1.2 to a concentration of ~2,632 U/ml. The amount of powder used to prepare the pepsin stock solution was calculated by using the purity-corrected pepsin specific activity value of 3404 U/mg. Activity was assessed using a pepsin activity assay (Section F.4.1).

F.3.2. Degradation of the Cry1B.868 and Cry1Da_7 Proteins by Pepsin

F.3.2.1. Degradation of the Cry1B.868 Protein by Pepsin

Degradation of the Cry1B.868 protein by pepsin was evaluated over time by analyzing samples collected at targeted incubation time points. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
0 min	0 min No Test Protein Control
0 min	0 min No Pepsin Control
0 min	Pepsin Treated T0
0.5 min	Pepsin Treated T1
2 min	Pepsin Treated T2
5 min	Pepsin Treated T3
10 min	Pepsin Treated T4
20 min	Pepsin Treated T5
30 min	Pepsin Treated T6
60 min	Pepsin Treated T7
60 min	60 min No Pepsin Control
60 min	60 min No Test Protein Control

The reaction mixture was prepared by adding 531.9 µl of pre-heated (38.3 ºC, 10 min) pepsin stock solution to a tube containing 50 µl of Cry1B.868 protein, which corresponded to 140 µg of Cry1B.868 protein and 1400 U of pepsin. The tube contents were mixed and immediately returned to the water bath (38.3 ºC). Samples (58.2 µl) were removed at 0.5, 2, 5, 10, 20, 30 and 60 min (corresponding to Pepsin Treated T1 through Pepsin Treated T7). Each 58.2 µl sample was placed immediately in a tube containing quenching mixture consisting of 20.4 µl of 0.7 M sodium

carbonate buffer and 19.6 µl of 5× loading buffer (5× LB; ~310 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8), heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

The Pepsin Treated T0 sample was prepared in a separate tube. The pepsin stock solution (53.2 µl) was quenched by the addition of 0.7 M sodium carbonate buffer (20.4 µl) and 5× LB (19.6 µl) and heated at 95-100 ºC for 5-10 min prior to the addition of the Cry1B.868 protein (5.0 µl).

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analysis.

F.3.2.2. Degradation of the Cry1Da_7 Protein by Pepsin

Degradation of the Cry1Da_7 protein by pepsin was evaluated over time by analyzing samples collected at targeted incubation time points. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
0 min	0 min No Test Protein Control
0 min	0 min No Pepsin Control
0 min	Pepsin Treated T0
0.5 min	Pepsin Treated T1
2 min	Pepsin Treated T2
5 min	Pepsin Treated T3
10 min	Pepsin Treated T4
20 min	Pepsin Treated T5
30 min	Pepsin Treated T6
60 min	Pepsin Treated T7
60 min	60 min No Pepsin Control
60 min	60 min No Test Protein Control

The reaction mixture was prepared by adding 683.9 µl of pre-heated (37.7ºC, 5 min) pepsin stock solution to a tube containing 50 µl of Cry1Da_7 protein, which corresponded to 180 µg of Cry1Da_7 protein and 1800 U of pepsin. The tube contents were mixed and immediately returned to the water bath (38.0ºC). Samples (73.4 µl) were removed at 0.5, 2, 5, 10, 20, 30 and 60 min (corresponding to Pepsin Treated T1 through Pepsin Treated T7). Each 73.4 µl sample was placed immediately in a tube containing quenching mixture consisting of 25.7 µl of 0.7 M sodium carbonate buffer and 24.8 µl of 5× loading buffer (5× LB; ~310 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8), heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

The Pepsin Treated T0 sample was prepared in a separate tube. The pepsin stock solution (68.4 µl) was quenched by the addition of 0.7 M sodium carbonate buffer (25.7 µl) and 5× LB (24.8 µl) and

heated at 95-100 ºC for 5-10 min prior to the addition of the Cry1Da_7 protein (5.0 µl). The sample was then heated again at 95-100 ºC for 5-10 min.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analysis.

F.3.2.3. Pepsin Degradation Experimental Controls

Experimental control samples were prepared to determine the stability of the Cry1B.868 and Cry1Da_7 proteins in 10 mM HCl, 2 mg/ml NaCl, pH ~1.2, the solution used to prepare the pepsin stock solution. The 0 min No Pepsin Control was prepared in a similar manner as described in Section F.3.2.2 for Pepsin Treated T0, except that 10 mM HCl, 2 mg/ml NaCl, pH 1.2 was added instead of the pepsin stock solution. The 60 min No Pepsin Control was prepared in a manner similar to 0 min No Pepsin Control, except the protein and 10 mM HCl, 2 mg/ml NaCl, pH 1.2 were incubated for 60 min at 38ºC before quenching with 0.7 M sodium carbonate buffer and 5× LB.

Experimental control samples were also prepared to determine the stability of the pepsin stock solution lacking the Cry1B.868 and Cry1Da_7 proteins. The 0 min No Test Protein Control was prepared in a similar manner as described in Section F.3.2.2 for Pepsin Treated T0, except that protein storage buffer (25 mM sodium carbonate, pH 10.5) was added in place of the Cry1B.868 and Cry1Da_7 proteins. The 60 min No Test Protein Control was prepared in a manner similar to 0 min No Test Protein Control, except that protein storage buffer and the pepsin stock solution were incubated for 60 min at 38ºC before quenching with 0.7 M sodium carbonate buffer and 5× LB.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analysis.

F.3.3. Preparation of the Pancreatin Stock Solution

Pancreatin contains a mixture of proteolytic enzymes and was prepared based on the method described in The United States Pharmacopoeia (USP, 1995). The pancreatin was obtained from Alfa Aesar (catalog number J62162) and was dissolved in 50 mM potassium phosphate buffer (pH 7.5) to a concentration of 10 mg of pancreatin powder/ml. Activity was assessed using a pancreatin activity assay (Section F.4.2).

F.3.3.1. Degradation of the Cry1B.868 and Cry1Da_7 Proteins by Pancreatin

F.3.3.1.1. Degradation of the Cry1B.868 Protein by Pancreatin

Degradation of the intact Cry1B.868 protein by pancreatin was evaluated over time by analyzing samples at multiple incubation time points. A numerical code using the numbers 0 through 8 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
0 min	0 min No Test Protein Control
0 min	0 min No Pancreatin Control
0 min	Pancreatin Treated T0
5 min	Pancreatin Treated T1
15 min	Pancreatin Treated T2
30 min	Pancreatin Treated T3
1 h	Pancreatin Treated T4
2 h	Pancreatin Treated T5
4 h	Pancreatin Treated T6
8 h	Pancreatin Treated T7
24 h	Pancreatin Treated T8
24 h	24 h No Pancreatin Control
24 h	24 h No Test Protein Control

The reaction mixture was prepared by adding 774.2 µl of pre-heated (37.7 ºC, 10 min) pancreatin stock solution to a tube containing 50 µl of the Cry1B.868 protein, corresponding to 140 µg of Cry1B.868 protein and 7,742 µg of pancreatin. The tube contents were vortex mixed and immediately returned to the water bath (37.6 ºC). Digestion samples (82.4 µl) were removed at 5, 15, 30 min, 1, 2, 4, 8, and 24 h (corresponding to time points Pancreatin Treated T1 through Pancreatin Treated T8) and immediately placed in a tube containing 26.1 µl of 5× LB, heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

The Pancreatin Treated T0 sample was prepared in a separate tube by first quenching 77.4 µl of pancreatin stock solution with 20.6 µl of 5× LB buffer and heating to 95-100 ºC for 5-10 min prior to the addition of 5.0 µl of the Cry1B.868 protein. Samples were heated again to 95-100 ºC for 5-10 min.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analysis.

F.3.3.1.2. Degradation of the Cry1Da_7 Protein by Pancreatin

Degradation of the intact Cry1Da_7 protein by pancreatin was evaluated over time by analyzing samples at multiple incubation time points. A numerical code using the numbers 0 through 8 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
0 min	0 min No Test Protein Control
0 min	0 min No Pancreatin Control
0 min	Pancreatin Treated T0
5 min	Pancreatin Treated T1
15 min	Pancreatin Treated T2
30 min	Pancreatin Treated T3
1 h	Pancreatin Treated T4
2 h	Pancreatin Treated T5
4 h	Pancreatin Treated T6
8 h	Pancreatin Treated T7
24 h	Pancreatin Treated T8
24 h	24 h No Pancreatin Control
24 h	24 h No Test Protein Control

The reaction mixture was prepared by adding 995.4 µl of pre-heated (37.8ºC, 5.5 min) pancreatin stock solution to a tube containing 50 µl of the Cry1Da_7 protein, corresponding to 180 µg of Cry1Da_7 protein and 9,954 µg of pancreatin. The tube contents were vortex mixed and immediately returned to the water bath (37.7ºC). Digestion samples (104.5 µl) were removed at 5, 15, 30 min, 1, 2, 4, 8, and 24 h (corresponding to time points Pancreatin Treated T1 through Pancreatin Treated T8) and immediately placed in a tube containing 26.1 µl of 5× LB, heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

The Pancreatin Treated T0 sample was prepared in a separate tube by first quenching 99.5 µl of pancreatin stock solution with 26.1 µl of 5× LB buffer and heating to 95-100 ºC for 5-10 min prior to the addition of 5.0 µl of the Cry1Da_7 protein. The sample was then heated again at 95-100 ºC for 5-10 min.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analysis.

F.3.3.2. Pancreatin Degradation Experimental Controls

Experimental control samples were prepared to determine the stability of the Cry1B.868 and Cry1Da_7 proteins in 50 mM KH_2PO_4, pH 7.5, the buffer used to prepare pancreatin stock solution. The 0 min No Pancreatin Control was prepared in a similar manner as described in Section F.3.3.1.2 for Pancreatin Treated T0, except that 50 mM KH_2PO_4, pH 7.5 was added instead of the pancreatin stock solution. The 24 hour No Pancreatin Control was also prepared in a similar manner, except the protein and 50 mM KH_2PO_4, pH 7.5 were incubated for 24 hour at 38ºC before quenching with 5× LB and heating.

Experimental control samples were also prepared to characterize the test system lacking the Cry1B.868 and Cry1Da_7 proteins. The 0 min No Test Protein Control was prepared in a similar manner as described in Section F.3.3.1.2 for Pancreatin Treated T0, except that protein storage buffer (25 mM sodium carbonate, pH 10.5) was added instead of Cry1B.868 and Cry1Da_7 proteins. The 24 hour No Test Protein Control was also prepared in a similar manner, except the protein storage buffer and pancreatin stock solution were incubated for 24 hour at 38ºC before quenching with 5× LB and heating.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analyzed.

F.3.4. Degradation of the Cry1B.868 and Cry1Da_7 Proteins by Pepsin Followed by Pancreatin

F.3.4.1. Degradation of the Cry1B.868 Protein by Pepsin Followed by Pancreatin

Degradation of the Cry1B.868 protein by sequential digestion was evaluated by first incubating the Cry1B.868 protein with pepsin for 2 min, quenching the pepsin degradation reaction, and then incubating the reaction mixture with pancreatin. The sequential digestion of the Cry1B.868 protein was evaluated over time by analyzing samples at multiple incubation time points with a numerical code using the number 0 through 7 to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
Pepsin Degradation	
0 min	0 min Pepsin Treated
2 min	2 min Pepsin Treated
Pancreatin Degradation	
0 min	SEQ 0 min No Test Protein Control
0 min	SEQ 0 min No Pancreatin Control
0 min	SEQ T0
0.5 min	SEQ T1
2 min	SEQ T2
5 min	SEQ T3
10 min	SEQ T4
30 min	SEQ T5
1 h	SEQ T6
2 h	SEQ T7
2 h	SEQ 2 h No Pancreatin Control
2 h	SEQ 2 h No Test Protein Control

In the pepsin degradation phase, 531.9 µl of pre-heated (38.4 ºC, 10 min) pepsin solution (2632 units of pepsin activity/ml) was added to 50 µl of the Cry1B.868 protein, corresponding to 140 µg of Cry1B.868 protein and 1,400 U of pepsin. The tube contents were mixed by vortexing and immediately returned to the water bath (38.3 ºC). The tube was removed at a targeted time of 2 min, and immediately quenched by adding 203.7 µl of 0.7 M sodium carbonate buffer. The 2 min Pepsin Treated sample was prepared by mixing 120 µl of the quenched sample with 30 µl of 5× LB, heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

In the pancreatin degradation phase, the quenched 2 min pepsin treated sample was digested in pancreatin. For this phase, 398.2 µl of pre-heated (38.3 ºC) pancreatin solution was added to 400 µl of quenched 2 min pepsin treated sample, corresponding to 72 µg total Cry1B.868 protein (based on predigested concentration) and 3,982 µg of pancreatin. The tube contents were mixed by vortexing and immediately returned to the water bath (38.3 ºC). Digestion samples (79.8 µl) were removed from the tube at targeted times of 30 sec, 2, 5, 10, 30 min, 1, and 2 h (corresponding to specimen codes SEQ T1 through SEQ T7) and immediately quenched by placing in a tube containing 20.0 µl of 5× LB, heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

The zero incubation time point for the pepsin degradation phase (0 min Pepsin Treated) was prepared in a separate tube. The pepsin solution (53.2 µl) was quenched by the addition of 0.7 M sodium carbonate buffer (20.4 µl) and 5× LB (19.6 µl) and heated at 95-100 ºC for 5-10 min prior to the addition of the Cry1B.868 protein (5.0 µl).

The zero incubation time point for the pancreatin digestion phase (SEQ T0) was prepared in a separate tube. The pancreatin solution (39.8 µl) was quenched with 5× LB (20.0 µl) and heated at 95-100 ºC for 5-10 min prior to the addition of the quenched 2 min pepsin treated Cry1B.868 (40.0 µl).

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analyzed.

F.3.4.2. Degradation of the Cry1Da_7 Protein by Pepsin Followed by Pancreatin

Degradation of the Cry1Da_7 protein by sequential digestion was evaluated by first incubating the Cry1Da_7 protein with pepsin for 2 min, quenching the pepsin degradation reaction, and then incubating the reaction mixture with pancreatin. The sequential digestion of the Cry1Da_7 protein was evaluated over time by analyzing samples at multiple incubation time points with a numerical code using the number 0 through 7 to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
Pepsin Degradation	
0 min	0 min Pepsin Treated
2 min	2 min Pepsin Treated
Pancreatin Degradation	
0 min	SEQ 0 min No Test Protein Control
0 min	SEQ 0 min No Pancreatin Control
0 min	SEQ T0
0.5 min	SEQ T1
2 min	SEQ T2
5 min	SEQ T3
10 min	SEQ T4
30 min	SEQ T5
1 h	SEQ T6
2 h	SEQ T7
2 h	SEQ 2 h No Pancreatin Control
2 h	SEQ 2 h No Test Protein Control

In the pepsin degradation phase, 683.9 µl of pre-heated (37.6ºC, 5 min) pepsin solution (2632 units of pepsin activity/ml) was added to 50 µl of the Cry1Da_7 protein, corresponding to 180 µg of Cry1Da_7 protein and 1,800 U of pepsin. The tube contents were mixed by vortexing and immediately returned to the water bath (37.7ºC). The tube was removed at a targeted time of 2 min, and immediately quenched by adding 256.9 µl of 0.7 M sodium carbonate buffer. The 2 min Pepsin Treated sample was prepared by mixing 120 µl of the quenched sample with 30 µl of 5× LB, heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

In the pancreatin degradation phase, the quenched 2 min pepsin treated sample was digested in pancreatin. For this phase, 497.7 µl of pre-heated (37.6ºC) pancreatin solution was added to 500 µl of quenched 2 min pepsin treated sample, corresponding to 90 µg total Cry1Da_7 protein (based on predigested concentration) and 4,977 µg of pancreatin. The tube contents were mixed by vortexing and immediately returned to the water bath (37.6ºC). Digestion samples (99.8 µl) were removed from the tube at targeted times of 30 sec, 2, 5, 10, 30 min, 1, and 2 h (corresponding to specimen codes SEQ T1 through SEQ T7) and immediately quenched by placing in a tube containing 25.0 µl of 5× LB, heated at 95-100 ºC for 5-10 min, and frozen on dry ice.

The zero incubation time point for the pepsin degradation phase (0 min Pepsin Treated) was prepared in a separate tube. The pepsin solution (68.4 µl) was quenched by the addition of 0.7 M sodium carbonate buffer (25.7 µl) and 5× LB (24.8 µl) and heated at 95-100 ºC for 5-10 min prior to the addition of the Cry1Da_7 protein (5.0 µl).

The zero incubation time point for the pancreatin digestion phase (SEQ T0) was prepared in a separate tube. The pancreatin solution (49.8 µl) was quenched with 5× LB (25.0 µl) and heated at 95-100 ºC for 5-10 min prior to the addition of the quenched 2 min pepsin treated Cry1Da_7 (50.0 µl).

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analyzed.

F.3.4.3. Pepsin Followed by Pancreatin Degradation Experimental Controls

The SEQ 0 min No Pancreatin Control was prepared in a similar manner as described for SEQ T0, except that 50 mM KH_2PO_4, pH 7.5 was added instead of pancreatin solution. The SEQ 2 hour No Pancreatin Control was also prepared in a similar manner, except the protein and 50 mM KH_2PO_4 were incubated for 2 hours at 38°C before quenching with 5× LB and heating.

The SEQ 0 min No Test Protein Control was prepared in a similar manner as described for SEQ T0, except that protein storage buffer (25 mM sodium carbonate, pH 10.5) was added instead of Cry1B.868 or Cry1Da_7 protein. The SEQ 2 hour No Test Protein Control was also prepared in a similar manner, except the protein storage buffer and pancreatin solution were incubated for 2 hours at 38ºC before quenching with 5× LB and heating.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analyzed.

F.4. Analytical Methods

F.4.1. Pepsin Activity Assay

Pepsin activity was determined by measuring the degradation of denatured hemoglobin. To conduct the assay, the pepsin stock solution (Section F.3.1), was diluted to 0.03 mg of powder per ml with 10 mM HCl, 2 mg/ml NaCl, pH ~1.2 [dilution factor (DF) = 25.67]. Five ml of acidified hemoglobin (2% (w/v)) was added to three test sample tubes and three blank tubes and all were pre-warmed for 5 min in a water bath at 37.7-38.0°C. Diluted pepsin stock solution (1 ml) was added to the three test sample tubes (test samples) and all six tubes were incubated for 10 min in the water bath. Ten ml of 5% (v/v) chilled trichloroacetic acid (TCA) was added to each of the six tubes and 1 ml diluted pepsin stock solution was then added to the three blank samples. Samples were mixed and then incubated for another 5 min in the water bath. Precipitated protein was removed from each sample using 0.45 µm syringe filters and the absorbance of the test and blank sample filtrates at 280 nm were measured using a SpectraMax M2 plate reader (Molecular Devices). One unit of pepsin produces a change in absorbance at 280 nm of 0.001 per min at 37 ± 2 ºC, pH 1.2. The units of pepsin in 1 ml of the stock solution were calculated using the following equation:

$$\frac{Mean\ Test_{A280nm} - Mean\ Blank_{A280nm}}{0.001\ \times\ 10\,\text{min}\ \times\ 1\,ml} \times DF$$

where 0.001 is the change in the absorbance at 280 nm per min produced by one unit of pepsin activity at 37 ± 2 ºC, pH 1.2; 10 min is the reaction time; 1 ml is the volume of the diluted pepsin stock solution added to the reaction; and DF is the dilution factor for the pepsin stock solution. The activity of pepsin was converted from units/ml to units/mg. Acceptable specific activity (units/mg pepsin powder) for the pepsin solution was equal to the specific activity determined by the manufacturer ± 1,000 units/mg.

F.4.2. Pancreatin Activity Assay

The pancreatin activity was determined by measuring the degradation of resorufin-labeled casein (Roche Life Science). To conduct the assay, the pancreatin stock solution (Section F.3.3), was diluted to 0.05× with 50 mM KH_2PO_4, pH 7.5. Fifty µl of 0.4% (w/v) resorufin-labeled casein and 50 µl of incubation buffer (200 mM Tris, pH 7.8, 20 mM $CaCl_2$) were added to three test sample tubes and three blank tubes and all were pre-warmed for 1.5-2.0 min in a water bath at 37.8°C. To initiate the reaction, 100 µl of 0.05× pancreatin stock solution was added to each of the three test sample tubes while 100 µl of 50 mM KH_2PO_4, pH 7.5 was added to each of the three blank tubes. All six tubes were incubated for 15 min in the water bath. Reactions were quenched by adding 480 µl of 5% (w/v) TCA to each tube, followed by vortex mixing. The samples were incubated in the water bath for an additional 10 min. The supernatants recovered after centrifugation (400 µl) were neutralized by the addition of 600 µl assay buffer (500 mM Tris-HCl, pH 8.8) and the absorbance of the test and blank supernatants was read at 574 nm using a SpectraMax M2 plate reader (Molecular Devices). One unit of pancreatin produces a change in the absorbance at 574 nm of 0.001 per min at 37 ± 2 ºC. The units of pancreatin in the stock solution were calculated using the following equation:

$$\frac{Mean\ Activity_{A574nm} - Mean\ Blank_{A574nm}}{0.001\ \times 15\,\text{min} \times\ 0.005\,ml}$$

where 0.001 is the change in the absorbance at 574 nm per min at 37 ± 2 ºC produced by one unit of pancreatin activity, 15 min is the reaction time, 0.1 ml is the volume of diluted pancreatin stock solution added to the reaction, and 0.005 ml is the volume of the pancreatin stock solution used in the assay (0.05× Pancreatin solution, 0.1 ml). An acceptable specific activity for the pancreatin stock solution was defined as 11,000 ± 3,000 U/ml.

F.4.3. SDS-PAGE and Colloidal Brilliant Blue G Staining

Pepsin-treated samples and associated control samples were subjected to SDS-PAGE using pre-cast Tricine 10-20% (w/v) polyacrylamide gradient mini-gels and Tricine SDS running buffer (Invitrogen). The Cry1B.868 and Cry1Da_7 proteins were loaded at 1 µg per lane based on total pre-digestion protein concentration. The experimental controls were loaded at the same volumes as those containing Cry1B.868 and Cry1Da_7 proteins so that they would be comparable. Mark12™ molecular weight marker (Invitrogen) was loaded to estimate the relative molecular

weight of proteins and peptides. Electrophoresis was performed at a constant voltage of 125-150 volts for 75-95 minutes. After electrophoresis, gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 25-60 min and stained for 18-20 hours in 1× Brilliant Blue G-Colloidal stain solution (Sigma), destained for 30 sec in 10% (v/v) acetic acid, 25% (v/v) methanol and then destained for 4.0-6 hours in a 25% (v/v) methanol solution. The gels were scanned using a Bio-Rad GS-900 densitometer (Bio-Rad) to produce digitized images to be used as figures for reporting purposes.

To estimate the LOD of the Cry1B.868 and Cry1Da_7 proteins, dilutions of the Pepsin Treated T0 samples were loaded on a second SDS-PAGE gel and the gel was run and processed exactly as the gel used to assess Cry1B.868 and Cry1Da_7 protein degradation in the presence of pepsin. Loads per lane were approximately 0.4 to 200 ng or 400 ng of the Cry1B.868 and Cry1Da_7, respectively. The LOD was determined as the lowest amount of the Cry1B.868 and Cry1Da_7 proteins that was visible on the gel with Colloidal staining.

Sequential digestion samples were also analyzed by SDS-PAGE as described above for pepsin degradation samples.

F.4.4. Western Blot Analysis

Pepsin-treated, pancreatin-treated, and sequential digestion samples, and the associated control samples, were subjected to SDS-PAGE using pre-cast Tricine 10-20% (w/v) polyacrylamide gradient mini-gels. The Cry1B.868 and Cry1Da_7 proteins were loaded at approximately 40 ng per lane based on total pre-digestion protein concentration. The experimental controls were loaded to equal the digestion samples. Electrophoresis was performed at a constant voltage of 125-150 volts for 75-95 minutes. After electrophoresis, the proteins were electrotransferred to PVDF membranes (Invitrogen). Pre-stained molecular weight markers (Precision Plus Protein™ Standards Dual Color Standard, Bio-Rad; MagicMark™ XP, Invitrogen) were used to verify electrotransfer of the proteins to the membranes and estimate the relative molecular weight of proteins. Proteins transferred to PVDF membranes were analyzed by western blot. The western blotting procedure was performed using an iBind™ Western System apparatus (Life Technologies).

F.4.4.1. Western Blot Analysis of Cry1B.868 Protein

The Cry1B.868 protein membranes were blocked with 1× iBind™ Solution (Life Technologies) and incubated with rabbit anti-Cry1B.868 antibody (lot 29121) at a dilution of 1:2,000 in 1× iBind™ Solution. After washing with 1× iBind™ Solution, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Vector Laboratories) at a dilution of 1:2,000 in 1× iBind™ Solution and washed again with 1× iBind™ Solution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare) and exposed to Hyperfilm™ ECL high performance chemiluminescence film (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica). The films were scanned using a Bio-Rad GS-900 densitometer to produce electronic images to be used as figures for reporting purposes.

To estimate the LOD of the Cry1B.868 protein, dilutions of either the Pepsin Treated T0 or the Pancreatin Treated T0 samples were subjected to western blot analysis run and processed exactly as the western blot used to assess Cry1B.868 protein degradation in the presence of pepsin or pancreatin, respectively. Loads per lane were approximately 0.08 to 40 ng of the Cry1B.868 protein. The LOD was determined as the lowest amount of the Cry1B.868 protein that was visible on the film.

F.4.4.2. Western Blot Analysis of Cry1Da_7 Protein

The Cry1Da_7 protein membranes were blocked with 1× iBind™ Solution (Life Technologies) and incubated with goat anti-Cry1Da_7 antibody (lot G1496) at a dilution of 1:1,000 in 1× iBind™ Solution. After washing with 1× iBind™ Solution, the membrane was incubated with horseradish peroxidase (HRP)-conjugated horse anti-goat IgG (Vector Laboratories) at a dilution of 1:1,000 in 1× iBind™ Solution and washed again with 1× iBind™ Solution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare) and exposed to Hyperfilm™ ECL high performance chemiluminescence film (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica). The films were scanned using a Bio-Rad GS-900 densitometer to produce electronic images to be used as figures for reporting purposes.

To estimate the LOD of the Cry1Da_7 protein, dilutions of either the Pepsin Treated T0 or the Pancreatin Treated T0 samples were subjected to western blot analysis run and processed exactly as the western blot used to assess Cry1Da_7 protein degradation in the presence of pepsin or pancreatin, respectively. Loads per lane were approximately 0.08 to 20 ng of the Cry1Da_7 protein. The LOD was determined as the lowest amount of the Cry1Da_7 protein that was visible on the film.

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Appendix G: Materials and Methods for Seed Germination and Dormancy Assessment of MON 95379

G.1. Materials

Seed germination and dormancy characteristics were assessed on seed from MON 95379 and the conventional control harvested from one 2018 field production site in Kihei, Hawaii, and on four reference maize hybrids obtained from commercial sources (Table G-1).

G.2. Characterization of the Materials

The presence of the MON 95379 event in the starting seed of the test material and the absence of the MON 95379 event in the starting seed of the conventional control were verified by event-specific polymerase chain reaction (PCR) analyses.

G.3. Germination Testing Facility and Experimental Methods

Germination and dormancy evaluations were conducted at SGS North America, Inc, Brookings, SD. The Principal Investigator was qualified to conduct seed germination and dormancy testing consistent with the standards established by the Association of Official Seed Analysts (AOSA, 2009; 2018a; 2018b).

The seed lots of MON 95379, the conventional control, and four reference hybrids were tested under two temperature regimes. The optimum temperature regime consisted of constant temperatures of approximately 25°C maintained for seven days (AOSA, 2018 a; b) . The suboptimum temperature regime consisted of constant temperatures of approximately 10°C for seven days followed by 25°C for four days (AOSA, 2009). One germination chamber was used for each temperature regime. Each chamber was maintained dark. The temperature inside each germination chamber was monitored and recorded throughout the duration of the study.

Rolled germination towels were assembled for each material by placing approximately 50 seeds on a pre-moistened and labeled germination towel. An additional pre-moistened germination towel was placed on top of the seed, and the towels and seed were rolled up. Approximately 100 seeds (a set of two germination towel rolls secured together with a rubber band) of each material were placed into a bucket to form a replication. Each temperature regime constituted a separate experiment that was conducted using a randomized complete block design with four replications (*i.e.*, buckets).

Each set of rolled germination towels in each temperature regime was assessed at prescribed timings for germinated, dead, firm swollen (viable and nonviable), and hard (viable and nonviable) seed. Additional details for each germination characteristic evaluated and the timing of evaluations are presented in Table VIII-1. Seeds placed under the optimum temperature regime were evaluated according to AOSA standards for testing of maize (AOSA, 2018a; b). Seeds placed under the suboptimum temperature regime were evaluated according to AOSA standards for cold testing of maize (AOSA, 2009).

Within both the optimum and suboptimum temperature regimes, firm-swollen and hard seeds remaining at the final evaluation date were subjected to a tetrazolium (Tz) test for viability according to AOSA standards (AOSA/SCST, 2010). The numbers of non-viable firm-swollen and non-viable hard seeds were added to the number of dead seed counted on all collection dates to determine the total number of dead seed. Total numbers of viable firm-swollen and viable hard seed were determined from the Tz test.

The percentage of seed in each assessment category was based on the number of seeds evaluated. Across temperature regimes, the total number of seeds evaluated from each set of rolled germination towels was approximately 100.

G.4. Statistical Analysis

For each temperature regime, ANOVA was conducted according to a randomized complete block design using SAS® (SAS, 2012) to compare MON 95379 to the conventional control for the germination and dormancy characteristics. If analysis of variance assumptions were not satisfied, Fisher's Exact test was conducted for that characteristic using SAS (SAS, 2012). The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$). The reference range for each characteristic was determined from the minimum and maximum mean values among the four references.

Table G-1. Starting Seed for Germination and Dormancy Assesment of MON 95379

Material Name	Regulatory Lot Number	Phenotype	Material Type
MON 95379	11493962	Insect Protected	Test
LH244+HCL617	11493959	Conventional	Control
Dekalb DKC65-18	11464652	Conventional	Reference
Dekalb DKC61-52	11479327	Conventional	Reference
Golden Harvest G07F23	11490232	Conventional	Reference
Mycogen 2H721	11490229	Conventional	Reference

Note: starting seed of test and control materials were produced at Kihei, HI. Reference materials were commercial sources.

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SAS. 2012. Software Release 9.4 (TS1M4). Copyright 2002-2012 by SAS Institute, Inc., Cary, North Carolina.

Appendix H: Material and Methods for Phenotypic, Agronomic, and Environmental Interaction Assessment of MON 95379 under Field Conditions

H.1. Materials

Agronomic, phenotypic, and environmental interaction characteristics were assessed for MON 95379, the conventional control, and reference hybrids grown under similar agronomic conditions. Four reference hybrids were planted per site (Table H-1). A total of 17 unique reference hybrids were evaluated among the eight sites.

H.2. Characterization of the Materials

The presence of the MON 95379 event in the starting seed of the test material and the absence of the MON 95379 event in the starting seed of the conventional control were verified by event-specific polymerase chain reaction (PCR) analyses.

H.3. Field Sites and Plot Design

Field trials in 2018 at eight sites provided a range of environmental and agronomic conditions representative of U.S. maize growing regions. The Principal Investigator at each site was familiar with crop growth and production and evaluation of crop characteristics. The study was established at each site in a randomized complete block design with four replications. Plot and row dimensions are listed in Table H-2.

H.4. Planting and Field Operations

Planting information, soil description, and cropping history of the sites are listed in Table H-2. The Principal Investigator at each site followed local agronomic practices including those related to seed bed preparation and trial maintenance such as application of agricultural chemicals, fertilizer, and irrigation. All maintenance operations were performed uniformly across all plots within each site.

Table H-1. Starting Seed for Phenotypic, Agronomic, and Environmental Interactions Assessment of MON 95379

Site Code[1]	Material Name	Regulatory Lot Number	Phenotype	Material Type
All	MON 95379	11479279	Insect Resistant	Test
All	LH244xHCL617	11479278	Conventional	Control
IAEH	Dekalb DKC61-52	11427255	Conventional	Reference
IAEH	Lewis 1407	11427264	Conventional	Reference
IAEH	Golden Harvest G12J11-A	11446928	Conventional	Reference
IAEH	LG Seeds LG2549	11446934	Conventional	Reference
IAOG	Dekalb DKC61-52	11427255	Conventional	Reference
IAOG	Dekalb DKC62-06	11427256	Conventional	Reference
IAOG	Golden Harvest G09C43	11446927	Conventional	Reference
IAOG	Agrigold A6267	11446941	Conventional	Reference
IARL	Lewis 1613	11427265	Conventional	Reference
IARL	Stone 5820	11427266	Conventional	Reference
IARL	Agrigold A6472	11446939	Conventional	Reference
IARL	Agrigold A6574	11446940	Conventional	Reference
ILHY	NH6769	11380173	Conventional	Reference
ILHY	Golden Harvest G09C43	11446927	Conventional	Reference
ILHY	LG Seeds LG2636	11446935	Conventional	Reference
ILHY	Agrigold A6472	11446939	Conventional	Reference
ILJO	Dekalb DKC64-85	11427257	Conventional	Reference
ILJO	Lewis 1613	11427265	Conventional	Reference
ILJO	Mycogen Seeds MY09V40	11446930	Conventional	Reference
ILJO	Agrigold A6472	11446939	Conventional	Reference
INSH	Kruger K-0708	11427267	Conventional	Reference
INSH	Mycogen Seeds 2H721	11446932	Conventional	Reference
INSH	LG Seeds LG2549	11446934	Conventional	Reference
INSH	LG Seeds LG2636	11446935	Conventional	Reference
NEYO	Dekalb DKC61-52	11427255	Conventional	Reference
NEYO	Dekalb DKC64-85	11427257	Conventional	Reference
NEYO	Golden Harvest G12J11-A	11446928	Conventional	Reference
NEYO	LG Seeds LG2549	11446934	Conventional	Reference
OHTR	Dekalb DKC62-06	11427256	Conventional	Reference
OHTR	Dekalb DKC64-85	11427257	Conventional	Reference
OHTR	Kruger K-0708	11427267	Conventional	Reference
OHTR	Golden Harvest G09C43	11446927	Conventional	Reference

Note: Starting seed of test and control materials were produced in Kihei, HI in 2017

[1] Site codes: IAEH = Shelby County, IA; IAOG = Boone County, IA; IARL = Jefferson County, IA; ILHY = Clinton County, IL; ILJO = Champaign County, IL; INSH = Clinton County, IN; NEYO = York County, NE; OHTR = Miami County, OH;

Table H-2. Field Information for Phenotypic, Agronomic, and Environmental Interactions Assessment of MON 95379

Site Code[1]	Planting Date[2]	Harvest Date[2]	Planting Rate (seeds/m^2)	Rows/ Plot	Inter-row Distance (m)	Row Length (m)	Plot Size (m^2)	Soil Texture	Organic Matter (%)	Previous Crop 2017
IAEH	05/18/2018	10/24/2018	9.5	8	76	6.1	37.2	Silty Clay Loam	3.1	Soybeans
IAOG	05/17/2018	10/23/2018	9.5	8	76	6.1	37.2	Loam	3.6	Soybeans
IARL	05/07/2018	09/25/2018	9.5	8	76	6.1	37.2	Silty Clay Loam	3.1	Soybeans
ILHY	05/22/2018	10/03/2018	9.5	8	76	6.1	37.2	Silt Loam	1.8	Soybeans
ILJO	05/17/2018	10/01/2018	8.6	8	76	6.2	38.1	Silty Clay Loam	4.7	Maize
INSH	05/08/2018	10/11/2018	9.5	8	76	6.1	37.2	Silty Clay Loam	3.4	Maize
NEYO	05/04/2018	10/17/2018	9.5	8	76	6.1	37.2	Silty Clay Loam	2.4	Soybeans
OHTR	05/09/2018	10/09/2018	9.5	8	76	6.4	39.0	Loam	2.3	Wheat

[1] Site codes: IAEH = Shelby County, IA; IAOG = Boone County, IA; IARL = Jefferson County, IA; ILHY = Clinton County, IL; ILJO = Champaign County, IL; INSH = Clinton County, IN; NEYO = York County, NE; OHTR = Miami County, OH.
[2] Date format = mm/dd/yyyy.

H.5. Phenotypic Characteristic Assessments

Phenotypic characteristics assessed and the timing of each assessment are listed in Table VII-1.

H.6. Environmental Interaction Assessments

The test, conventional control, and commercial references were evaluated at each site for differences in plant responses to abiotic stressors, diseases, and arthropod pests. Evaluations were performed four times during the growing season at the following growth stages: V5 – V8, V12 – R1, R1 – R3, and R4 – R5.

The Field Co-operator at each site identified abiotic stressors, diseases, and arthropod pests that were either actively causing plant injury in the plots or likely to occur in maize during a given observation period. Recognizing that damage symptoms for certain stressors can be similar and sometimes difficult to identify to specific taxonomic levels, Field Cooperators experienced in agricultural research identified stressors to the best of their ability based on knowledge of what would most likely be occurring at that site and at that time of the season. Stressors assessed often varied among observations and sites.

Ratings were made using the categorical scale of increasing severity listed below:

Category	Severity of plant damage
None	No symptoms observed
Slight	Symptoms not damaging to plant development (*e.g.,* minor feeding or minor lesions); mitigation likely not required
Moderate	Intermediate between slight and severe; likely requires mitigation[1]
Severe	Symptoms damaging to plant development (*e.g.,* stunting or death); mitigation unlikely to be effective

[1]Pest management is based on pest pressure threshold to represent commercial crop production practices that would produce an agronomically acceptable crop (Appendix L).

H.7. Statistical Analysis/Data Summarization

A combined-site analysis was conducted according to a randomized complete block design using SAS® (SAS, 2012) to compare the test to the conventional control for the phenotypic characteristics listed in Table VIII-1. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$). Descriptive statistics are provided for one characteristic, seed loss, that had insufficient variability for formal statistical analysis. The reference range for each phenotypic characteristic was determined from the minimum and maximum mean values among the 17

references, where each mean was combined over all the sites at which the reference was planted. There were no plots for which data were excluded from the study.

The environmental interactions data consisting of plant responses to abiotic stressors, diseases, and arthropod pests are categorical and were considered different in susceptibility or tolerance if the range of injury symptoms did not overlap between the biotechnology-derived crop and the conventional control across all four replications within an observation (see Appendix L for additional context regarding assessment of potential differences between the test and conventional control).

H.8. Detailed Results for Environmental Interactions Assessments for MON 95379

No differences were observed between the test and the conventional control for any of the 96 comparisons for the assessed abiotic stressors: cold temperatures, excessive rain, hail, high temperatures, high winds, mineral toxicity, nutrient deficiency, soil compaction, and sun scald (Table H-3).

No differences were observed between the test and the conventional control for any of the 96 comparisons for the assessed arthropod pests: aphids, billbugs, corn flea beetles, corn rootworm beetles, grape colaspis, grasshoppers, Japanese beetles, leafhoppers, sap beetles, slugs, spider mites, stink bugs, and thrips (Table H-4).

No differences were observed between the test and the conventional control for any of the 96 comparisons for the assessed diseases: anthracnose, common rust, common smut, corn stunt, ear root, eyespot, Goss's bacterial wilt, gray leaf spot, northern leaf blight, northern leaf spot, physoderma brown spot, southern rust, stalk root, and Stewart's wilt (Table H-5).

The results of the assessed environmental interactions support the conclusion that MON 95379 is not expected to pose increased plant pest potential compared to conventional maize.

Table H-3. Summary of Abiotic Stressor Response Evaluations for MON 95379 and the Conventional Control in 2018 U.S. Field Trials

Abiotic Stressor	Total Observations across Sites	Number of Observations without Differences between the Test and the Conventional Control
Total	96	96
Cold Temperatures	3	3
Excessive Rain (i.e. waterlogging)	14	14
Hail	13	13
High Temperatures	10	10
High Winds (lodged plants)	25	25
Mineral toxicity	2	2
Nutrient deficiency	15	15
Soil compaction	8	8
Sun scald	6	6

Notes: No differences were observed between the test and the conventional control during any observation for responses to any of the assessed abiotic stressors. Categorical data were summarized across sites and observation times.

Table H-4. Summary of Arthropod Damage Evaluations for MON 95379 and the Conventional Control in 2018 U.S. Field Trials

Arthropod	Total Observations across Sites	Number of Observations without Differences between the Test and the Conventional Control
Total	96	96
Aphids (Aphididae)	15	15
Billbugs (Curculionidae)	1	1
Corn flea beetles (Chrysomelidae)	10	10
Corn rootworm beetles (Chrysomelidae)	13	13
Grape colaspis (Chrysomelidae)	2	2
Grasshoppers (Acrididae)	19	19
Japanese beetles (Scarabaeidae)	14	14
Leafhoppers (Cicadellidae)	4	4
Sap beetles (Nitidulidae)	2	2
Slugs[1]	1	1
Spider mites (Tetranychidae)	6	6
Stink bugs (Pentatomidae)	8	8
Thrips (Thripidae)	1	1

Notes: No differences were observed between the test and the conventional control during any observation for damage caused by any of the assessed arthropods. Categorical data were summarized across sites and observation times.

[1] Slugs are not arthropods but are occasional pests in maize

Table H-5. Summary of Disease Damage Evaluations for MON 95379 and the Conventional Control in 2018 U.S. Field Trials

Disease	Total Observations Across Sites	Number of Observations Without Differences Between MON 95379 and the Conventional Control
Total	96	96
Anthracnose (*Colletotrichum graminicola*)	13	13
Common rust of maize (*Puccinia sorghi*)	16	16
Common smut of maize (*Ustilago maydis*)	9	9
Corn stunt (*Spiroplasma kunkelii*)	3	3
Eyespot (*Aureobasidium zeae*)	9	9
Goss's bacterial wilt (*Clavibacter michiganensis* subsp. *nebraskensis*)	4	4
Gray leaf spot (*Cercospora zeae-maydis*)	19	19
Northern leaf blight of maize (*Exserohilum turcicum*)	15	15
Northern leaf spot (*Bipolaris zeicola*)	2	2
Physoderma brown spot (*Physoderma maydis*)	1	1
Southern rust of maize (*Puccinia polysora*)	1	1
Stalk Rot (includes *Colletotrichum graminicola*, *Erwinia chrysanthemi* pv. *zeae*, *Macrophomina phaseolina*, *Stenocarpella maydis*, *Fusarium* spp., *Pythium aphanidermaturm*)	2	2
Stewart's wilt (*Pantoea stewartii*)	2	2

Notes: No differences were observed between the test and the conventional control during any observation for damage caused by any of the assessed diseases. Categorical data were summarized across sites and observation times.

References for Appendix H

SAS. 2012. Software Release 9.4 (TS1M4). Copyright 2002-2012 by SAS Institute, Inc., Cary, North Carolina.

Appendix I: Materials and Methods for Compositional Analysis of MON 95379 Maize Grain and Forage

Compositional comparisons between MON 95379 and the conventional control maize hybrid were performed using the principles and analytes outlined in the OECD consensus document for maize composition (OECD, 2002). These principles are accepted globally and have been employed previously in assessments of maize products derived through biotechnology. The compositional assessment was conducted on grain and forage samples harvested from multiple U.S. field sites during 2018 grown under typical agronomic field conditions practices.

I.1. Materials

Harvested grain and forage from MON 95379 and a conventional control that has similar genetic background to that of MON 95379 hybrid were compositionally assessed.

I.2. Characterization of the Materials

The identities of MON 95379 and the conventional control were confirmed prior to use in the compositional assessment.

I.3. Field Production of the Samples

Grain and forage samples were harvested from MON 95379 and the conventional control grown in the United States during the 2018 season. The field production was conducted at five sites. The field sites were planted in a randomized complete block design with four replicates per site. MON 95379 and the conventional control were grown under normal agronomic field conditions for their respective growing regions.

Grain was harvested at physiological maturity and shipped at ambient temperature from the field sites to Bayer. Forage was harvested at R5 and shipped on dry ice from the field sites to Bayer. A subsample for compositional analysis was obtained from each tissue sample collected. These subsamples were ground and stored in a freezer set to maintain –20°C until their shipment on dry ice to Eurofins Food Integrity & Innovation Laboratories Inc. (Madison, Wisconsin) for analysis.

I.4. Summary of Analytical Methods

Nutrients analyzed in this study included protein, total fat, ash, amino acids, linoleic acid, carbohydrates by calculation, fiber (acid detergent fiber (ADF), neutral detergent fiber (NDF)) in the grain, protein, total fat, ash, carbohydrates by calculation and fiber (ADF and NDF) in the forage. The anti-nutrients assessed in grain included phytic acid and raffinose.

All compositional analyses were performed at Eurofins Food Integrity & Innovation Laboratories, Inc. (Madison, Wisconsin). Methods for analysis were based on internationally-recognized

procedures and literature publications. Brief descriptions of the methods utilized for the analyses are described below.

I.5. Acid Detergent Fiber

Sample aliquots were weighed into pre-weighed filter bags. Samples were placed in an ANKOM Fiber Analyzer and treated with an acid detergent solution containing sulfuric acid with cetyl trimethylammonium bromide, then filtered to remove proteins, starches, simple sugars, pectins, and ash. Fats and pigments were removed via an acetone wash leaving cellulose and lignin fractions. The remaining residue was the acid detergent fiber and was determined gravimetrically. The results are reported on a fresh weight (fw) basis. The limit of quantitation was calculated as 1.00%.

I.6. Amino Acids

The following 10 amino acids were analyzed:

Total arginine	Total methionine
Total histidine	Total phenylalanine
Total isoleucine	Total threonine
Total leucine	Total tryptophan
Total lysine	Total valine

The samples were hydrolyzed in 6N hydrochloric acid for approximately 24 hours at approximately 106-118ºC. Tryptophan was hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for approximately 20 hours.

The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection. The amino acids were quantified using external standards. The results are reported on a fresh weight basis. The limit of quantitation for this study was 0.100 mg/g.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
L-Arginine Monohydrochloride	Sigma-Aldrich	SLBT2948	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	SLBS4669	100
L-Isoleucine	Sigma-Aldrich	SLBS6030	100
L-Leucine	Sigma-Aldrich	SLBS4968	100
L-Lysine Monohydrochloride	Sigma-Aldrich	SLBM5436V	100
L-Methionine	Sigma-Aldrich	SLBS7026	100
L-Phenylalanine	Sigma-Aldrich	SLBQ7928V	100
L-Threonine	Sigma-Aldrich	SLBT5994	100
L-Tryptophan	Sigma-Aldrich	BCBT8640	100
L-Valine	Sigma-Aldrich	SLBT4145	99

I.7. Ash

All organic matter was driven off when the samples were ignited at approximately 550ºC in a muffle furnace for at least 5 hours. The remaining inorganic material was determined gravimetrically and referred to as ash. The results are reported on a fresh weight basis. The limit of quantitation was 0.100%.

I.8. Carbohydrate

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation:

$$\% \text{ carbohydrates} = 100\ \% - (\% \text{ protein} + \% \text{ fat} + \% \text{ moisture} + \% \text{ ash})$$

The limit of quantitation was calculated as 0.100% on a fresh weight basis.

I.9. Fat by Acid Hydrolysis

The samples were hydrolyzed with hydrochloric acid. The fat was extracted using ether and hexane. The extracts were dried down and filtered through a sodium sulfate column. The remaining extracts were then evaporated, dried, and weighed. The limit of quantitation was calculated as 0.100% on a fresh weight basis.

I.10. Fat by Soxhlet Extraction

The samples were weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the samples to remove the fat. The extract was then

evaporated, dried, and weighed. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.100%.

I.11. Linoleic Acid

The lipid was extracted and saponified with 0.5N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The results were converted to their triglyceride equivalent and reported on a fresh weight basis. The Methyl Linoleate analytical reference standard was purchased from Nu-Chek Prep. The lot numbers were Z-2 and X-2 with a purity of 99.8%. The limit of quantitation was calculated as 0.00245%.

I.12. Moisture

The samples were dried in a vacuum oven at approximately 100°C. The moisture weight loss was determined and converted to percent moisture. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.100%.

I.13. Neutral Detergent Fiber

Sample aliquots were weighed into pre-weighed filter bags. Samples were placed in an ANKOM Fiber analyzer and treated with a neutral detergent solution containing EDTA. Samples were then filtered to remove proteins, simple sugars, pectins, and ash. Fats and pigments were removed via an acetone wash leaving hemicellulose, cellulose, and lignin fractions. Starches were removed with a heat stable alpha amylase soak. The remaining residue was the neutral detergent fiber and was determined gravimetrically. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 1.00%.

I.14. Phytic Acid

The samples were extracted using hydrochloric acid and sonication, purified using a silica-based anion exchange column, concentrated and injected onto a high-performance liquid chromatography (HPLC) system with a refractive index detector. The Phytic Acid Sodium Salt Hydrate analytical reference standard was purchased from Sigma-Aldrich. The lot number was BCBQ7037V with a purity of 79.909%. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.125%.

I.15. Protein

The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. Instrumentation was used to automate the digestion, distillation and titration processes. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.100%.

I.16. Raffinose

Sugars in the samples were extracted with a 50:50 water: methanol solution. Aliquots were taken, dried under inert gas, and then reconstituted with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucopyranoside as the internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid treatment, and then analyzed by gas chromatography using a flame ionization detector. The D-(+)-Raffinose pentahydrate analytical reference standard was purchased from Sigma-Aldrich. The lot number was WXBC5581V with a purity of 99.1%. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.0500%.

I.17. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Bayer for review. Data were then transferred to the Bayer Regulatory Statistics Team where they were converted into the appropriate units and statistically analyzed. The following formulas were used for re-expression of composition data for statistical analysis (Table I-1):

Table I-1. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Proximates, Fiber, Phytic Acid, Raffinose	% fw	% dw	X/d
Amino Acids (AA)	mg/g fw	% dw	X/(10d)
Fatty Acids (FA), Linoleic Acid	% fw	% Total FA	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA

[1]'X' is the individual sample value; d is the fraction of the sample that is dry matter.

In order to complete a statistical analysis for a compositional constituent in this compositional assessment, at least 50% of all the values for an analyte in grain or forage had to be greater than the assay limit of quantitation (LOQ). No analytes with more than 50% of observations below the assay LOQ were observed.
The following linear mixed model was used for the combined-site analysis.

$$Y_{ijk} = \mu + S_i + R(S)_{j(i)} + M_k + (SM)_{ik} + \varepsilon_{ijk} \quad (1)$$

where:
Y_{ijk} is the observed response for the k^{th} substance in the j^{th} replicate of the i^{th} site;
μ is the overall mean;
S_i is the random effect of the i^{th} site;
$R(S)_{j(i)}$ is the random effect of the j^{th} replicate nested with the i^{th} site;
M_k is the fixed effect of the k^{th} substance;
$(SM)_{ik}$ is the random effect of the interaction between the i^{th} site and k^{th} substance;
ε_{ijk} is the residual error.
SAS PROC MIXED was used to fit model (1) separately for each component to conduct the statistical analysis. Studentized residuals were obtained to detect potential outliers in the dataset. Studentized residuals tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Data points that are outside of the ± 6 studentized residual ranges are considered as potential outliers. No value had a studentized residual outside of the ± 6 range for this study.

The linear mixed model (1) assumes that the experimental errors, ε_{ijk}, are independent, normally-distributed, and have a common variance. In this analysis, independence of the errors was controlled by the randomized complete block design. The normality and common variance assumptions were checked by visual examination of residual plots and histograms. No extreme violations were observed for any characteristic. Comparisons between MON 95379 and conventional control were defined within the model (1) and tested using t-tests.

References for Appendix I

OECD. 2002. Consensus document on compositional considerations for new varieties of maize (*Zea mays*): Key food and feed nutrients, anti-nutrients and secondary plant metabolites. OECD ENV/JM/MONO (2002)25. Organisation of Economic Co-Operation and Development, Paris, France.

Appendix J: Materials, Methods, and Results for Tier I NTO Testing

J.1. Evaluation of the Potential for Interaction between the Cry1Da_7 and Cry1B.868 Proteins Expressed in MON 95379 with Fall Armyworm (*Spodoptera frugiperda*)

Introduction

The stacking of Cry proteins that confer distinct modes of action in combined trait products is an essential tool to help to control key insect pests while delaying the onset of insect resistance. Evaluation of the potential interaction between multiple Cry proteins is conducted as an early step of the safety assessment for Cry proteins produced by conventional breeding or as a vector stack. In 2009, EPA published a codified framework and data requirements for assessing the safety of combined trait products (U.S. EPA, 2009). These data are used to bridge to the existing risk assessments for the individual Cry proteins. The first two requirements are to demonstrate that the composition of the inserted genetic material is unchanged and the expression (i.e., exposure) level Cry proteins in the single and combined trait products are comparable. Neither of these data requirements apply to MON 95379 maize as both proteins are expressed as a vector stack.

The third component of a combined trait bridging assessment is to assess for a potential toxicological interaction between the Cry proteins in a sensitive species (U.S. EPA 2009). Historically, EPA has referred to this study as the "synergy" study. The term "synergism" can sometimes have negative or confounding connotations in other applications; therefore, the more precise term "Greater than Additive" (GTA) will be used. Additionally, the term "Less than additive" (LTA) interaction will be used (e.g., 5 + 5 = 1) rather than the term antagonism and does not constitute a safety concern for Cry proteins. A GTA interaction (e.g., 5 + 5 = 50) could impact conclusions of the risk assessment and if the level of GTA activity is sufficiently high, additional testing with a mixture of the Cry proteins could be required. Recommended guidance from EPA indicates that only levels of combined activity in a sensitive species >10-fold above that expected through additivity would constitute a need for additional testing in non-target organisms (EPA, 2009). This recommended threshold is based on margins of exposure ≥10 that are generally achieved in NTO testing.

The design of an interaction study with two proteins should be based on the null hypothesis of no interaction. Results from resistant insect bioassays, disabled insecticidal protein bioassays, and cell-based assays using insect cell lines expressing individual receptors indicate that CryB.868 and Cry1Da_7 utilize receptors that are distinct from each other as well as other commercialized *Bt* proteins (Wang et al., 2019). This makes Cry1B.868 and Cry1Da_7 amenable to use in combined trait products both together and with other commercialized *Bt* proteins making them a potentially valuable tool for Insect Resistance Management programs. While Wang et al. does not directly address the potential for GTA activity in a sensitive species, the results demonstrate that Cry1B.868 and Cry1Da_7 act through distinct receptors supporting the hypothesis that Cry1B.868

and Cry1Da_7 act would be expected to act independently. Therefore, when expressed together in MON 95379, their activities would be predicted to be additive against a species that is susceptible to both proteins (e.g., 5 + 5 = 10).

The chosen test species for an interaction study must be sensitive to at least one protein with the underlying notion that an interaction between the two proteins is most likely to be observed in a sensitive species where responses can be quantifiably measured. This approach is consistent with the expectation that for NTOs, where exposure levels for proteins with different modes of action (i.e., act independently) are below those needed to elicit adverse effects, a response from the combination of proteins is not predicted (e.g., 0 + 0 = 0 or NOEC + NOEC = NOEC; EPA 2009; Levine and Borgert, 2018). However, if the margins of exposure for an NTO are low, (e.g., effects observed at near field expression levels), and a GTA response is observed in a sensitive species, then additional studies may be needed to evaluate the impact of a mixture of both proteins on that NTO species.

To assess for a potential interaction between Cry1B.868 and Cry1Da_7, Fall armyworm (FAW) was selected as a test species because it is sensitive to both Cry1B.868 and CryDa_7 in laboratory diet-incorporation bioassays. In the interaction study described below, the 50% effect levels for Cry1B.868 and Cry1Da_7 alone and in combination were evaluated and their combined predicted activity was calculated with the concentration addition model. The concentration addition model provides results generally similar to other mixture models like response addition, but in some circumstances, can be more conservative by estimating higher combined activity (Finney, 1971; Levine and Borgert, 2018). Therefore, the concentration addition model is EPA's default model for evaluation of mixtures of toxicants with similar modes of action (EPA, 1986, Tabashnik et al., 1994).

Using growth-inhibition as a sensitive endpoint with FAW, the concentration addition model is given by:

$$\frac{1}{Predicted\ GI_{50}} = \frac{\pi a}{GI_{50}a} + \frac{\pi b}{GI_{50}b}$$

πa and πb are the proportions of the relative expression of Cry1B.868 and Cry1Da_7 proteins in MON 95379 maize tissue.

$GI_{50}a$ and $GI_{50}b$ represent the observed GI_{50} values obtained with the Cry1B.868 and Cry1Da_7 single-expressing lines.

If the predicted combined activity (GI_{50}) falls within the 95% confidence interval (CI) of the observed combined activity for Cry1B.868 and Cry1Da_7 it can be concluded that combined activity is consistent with additivity. Interpretation of mixture toxicity calculations are commonly based on constructing a CI around the fitted effect of the response and then analyzing whether the predicted effect is captured by this CI (Tabashnik, 1992; Kudsk and Mathiassen, 2004; Levine and Borgert, 2018; Levine et al., 2016; Levine et al., 2019). A CI quantifies the degree of uncertainty around an estimate of a predicted or observed endpoint of interest. The advantage of this approach is that it considers the uncertainty in the predicted and/or observed response.

Results of the interaction study indicate that the combined activity of Cry1B.868 and Cry1Da_7 is additive, and this conclusion is consistent with the other lines of evidence showing that the two proteins act independently. Details of the study design, analysis and interpretation in context of the NTO assessment are provided below. Finally, additive activity of Cry1B.868 and Cry1Da_7 are conservatively assessed with a supplementary Hazard Index analysis adding together risk quotients from the independent assessments for each protein. The results of this analysis confirm the conclusion that adverse effects on NTOs from exposure to Cry1B.868 and Cry1Da_7 at the levels expressed in MON 95379 maize are not anticipated.

Purpose

The purpose of this study was to evaluate the potential for a toxicological interaction between the lepidopteran-active Cry1B.868 and Cry1Da_7 proteins in MON 95379.

Experimental Design

The study was designed to test the null hypothesis of no interaction between Cry1B.868 and Cry1Da_7 and was assessed using a concentration addition model. Cry protein expression was determined in tissues collected from Cry1B.868 and Cry1Da_7 single events as well as MON 95279 tissues using a quantitative ELISA. This analysis was conducted such that activities of Cry1B.868 and Cry1Da_7 could be expressed with respect to protein concentration to determine

the relative proportions of Cry1B.868 and Cry1Da_7 expressed in MON 95379 tissue. The activity of the Cry1B.868 and Cry1Da_7 in single event tissues as well as MON 95379 tissue was assessed with diet-incorporation bioassays using the sensitive species, FAW. The bioassay was repeated on three separate days using different batches of insects. The predicted combined activity was calculated based on the activity of Cry1B.868 and Cry1Da_7 single events and was compared to the observed activity of MON 95379 tissue. Specifically, the predicted combined activity of Cry1B.868 and Cry1Da_7 was compared to the 95% confidence interval for the observed activity of MON 95379 maize to determine whether to reject the null hypothesis of no interaction.

Test Materials

The test and control substances were finely-ground lyophilized tissue from the materials listed in Table J-1. Leaf tissue was harvested when all plants reached the V8 growth stage, and then stored in a -80 °C freezer before and after processing.

Table J-1. Summary of test and control substances

Substance Name (Treatment)[1]	IP Protein[2]	Phenotype
MON 95379	Cry1B.868 & Cry1Da_7	Insect-protected
Cry1B.868-single	Cry1B.868	Insect-protected
Cry1Da_7-single	Cry1Da_7	Insect-protected
Conventional Control (Assay Control)	None	Conventional

[1] All test and control substances have a common germplasm.
[2] IP = Insect-protected

J.1.1. Protein Expression Analysis and Tissue Preparation

Protein levels in MON 95379, the Cry1B.868-single, and Cry1Da_7-single tissues were quantified using ELISA. These values were used to convert the tissue amounts of each test and control treatment into µg of total protein/mL diet. Additionally, the ratio of each individual protein in MON 95379 was used in the concentration addition model to calculate the predicted GI_{50} value for the combined trait product. The protein analysis data summary results are provided in Table J-2. Since lyophilized tissues were used in this study, the fresh weight values determined by ELISA were converted into dry weight values (Table J-2). Tissue samples utilized in this study were primary milled, freeze dried, and ground again prior to study initiation.

TableJ-2. Summary of protein levels in leaf tissue with the fresh weight values converted to dry weight values.

Protein	Material Name	Mean *Bt* Concentration (µg/g fwt)	Moisture Conversion Factor for OSL-1 Corn Leaf Tissue	Mean *Bt* Concentration (µg/g dwt)
Cry1B.868	Conventional	<LOQ[1]	0.15	<LOQ
	Cry1Da_7-single	<LOQ[1]	0.15	<LOQ
	Cry1B.868-single	81	0.15	540
	MON 95379	86	0.15	573
Cry1Da_7	Conventional	<LOQ[1]	0.15	<LOQ
	Cry1Da_7-single	4.7	0.15	31
	Cry1B.868-single	<LOQ[1]	0.15	<LOQ
	MON 95379	17	0.15	113

[1]LOQ = Limit of quantitation

J.1.2. FAW Bioassay Testing Procedure

Prior to the start of the assay, FAW eggs were placed into covered hatch-boxes and held at a target temperature of 10°C prior to being incubated for hatching at a target temperature of 27°C. A diet-incorporation insect bioassay procedure was used to characterize the biological activity of the treatments shown in Table J-1.

Treatments were prepared by mixing a dosing solution that contained finely-ground lyophilized leaf tissue and purified water with an agar-based multiple-species diet (Southland) to achieve a final volume and vortex-mixed until visually homogenous. A repeat pipettor was used to dispense 16, 1.0 mL aliquots of each treated diet mixture into individual wells of 128-well bioassay trays. The diet was allowed to cool and solidify before adding one newly hatched FAW to each well. Each bioassay replicate contained a target number of 16 individually housed FAW larvae per treatment which were covered with a ventilated adhesive cover. Three conventional control treatments, each containing 10.0 mg of conventional control tissue/ml of diet, were included with each of three bioassays replicates. The treatment concentrations were chosen to appropriately characterize concentration-response relationships for each of the test substances. The original treatment concentrations were mg tissue/mL diet. However, prior to statistical analysis, the

concentrations for each treatment were converted to µg *Bt* protein/mL diet using the dry-weight converted values from the tissue expression analysis (Table J-2 and Table J-3). For the MON 95379 treatments, six diet concentrations (0.054, 0.11, 0.21, 0.43, 0.86 and 1.72 µg total *Bt* protein (Cry1B.868 and Cry1Da_7)/mL diet) were used, for the Cry1B.868-single treatment, six diet concentrations (0.042, 0.084, 0.16, 0.34, 0.68, and 1.35 µg Cry1B.868/mL diet) were used, and for the Cry1Da_7-single treatment, six diet concentrations (0.0097, 0.019, 0.039, 0.078, 0.16 and 0.31 µg Cry1Da_7/mL diet) were used in each of the three bioassay replicates. To ensure each treatment level replicate contained an approximately equal amount of tissue, conventional control tissue was added to each treatment level as necessary to obtain a final tissue concentration of approximately 10.0 mg tissue/mL of diet. Bioassay trays were incubated at a target temperature of 27°C, 60% relative humidity and a 14h:10h light:dark photoperiod. The bioassay was replicated three times on separate days using separate batches of insects.

At the end of each seven-day incubation period for each bioassay replicate, the actual number of larvae infested, the number of surviving larvae, and the combined larval mass of the surviving insects were recorded for each treatment concentration.

For each bioassay replicate, the test and control treatments were run concurrently on the same day with the same batch of insects. The bioassay was replicated three times on separate days with separate batches of insects. The bioassay was considered valid if the mean mortality was <20% and the calculated GI_{50} for each treatment group was bracketed by at least one treatment level.

Table J-3. Summary of protein levels in leaf tissue with the fresh weight values converted to dry weight values.

Treatment Description	mg of Tissue (dwt)/ml of Diet	Mean *Bt* Concentration in Tissue (µg/mg dwt)[1]	µg *Bt* Protein/ml of Diet
MON 95379 Tissue	0.078	0.686	0.054
MON 95379 Tissue	0.156	0.686	0.11
MON 95379 Tissue	0.313	0.686	0.21
MON 95379 Tissue	0.625	0.686	0.43
MON 95379 Tissue	1.25	0.686	0.86
MON 95379 Tissue	2.5	0.686	1.72
Cry1B.868-single Tissue	0.078	0.540	0.042
Cry1B.868-single Tissue	0.156	0.540	0.084
Cry1B.868-single Tissue	0.313	0.540	0.16
Cry1B.868-single Tissue	0.625	0.540	0.34
Cry1B.868-single Tissue	1.25	0.540	0.68
Cry1B.868-single Tissue	2.5	0.540	1.35
Cry1Da_7-single Tissue	0.313	0.031	0.0097
Cry1Da_7-single Tissue	0.625	0.031	0.019
Cry1Da_7-single Tissue	1.25	0.031	0.039
Cry1Da_7-single Tissue	2.5	0.031	0.078
Cry1Da_7-single Tissue	5.0	0.031	0.16
Cry1Da_7-single Tissue	10	0.031	0.31

[1]Mean *Bt* concentration in MON 95379 is the sum of Cry1B.868 and Cry1Da_7 in MON 95379 from Table J-2.

J.1.3. Data Analysis and Interpretation

GI_{50} values for the concentration-response of the test substances were estimated along with their 95% CIs by logistic regression (Table J-5). The data from the three replicate bioassays performed on separate days was analyzed in a combined analysis.

Under the concentration addition model, the activity of Cry1B.868 ($GI_{50}a$) and Cry1Da_7 ($GI_{50}b$) were added to predict the activity of MON 95379 (Finney 1971; Tabashnik 1992). The predicted GI_{50} values were calculated as follows: 1/predicted $GI_{50} = \pi a/GI_{50}a + \pi b/GI_{50}b$, where πa and πb are the proportions of the two single trait products expressed in MON 95379. Using the expression data in Table J-3 to convert from mg tissue /ml diet to ug *Bt* protein/ml diet the proportions of the single proteins in the combined trait were calculated as:

Table J-4. Relative Concentrations of Cry1B.868 and Cry1Da_7 in Lyophilized Leaf Tissue from MON 95379 Maize

Protein Type	Protein Expression (µg/g dwt)	Proportion
Cry1B.868	573	πa = 573/(573+113) or 0.835
Cry1Da_7	113	πb = 113/(573+113) or 0.165

Results

Bioassay replicates 1 and 2 both had no mortality and replicate 3 had two dead insects out of 45 infested for 4.4% mortality thereby meeting the acceptance criteria of less than or equal to 20% mean mortality for the assay control treatment. All treatments in all replicates had at least one growth inhibition response between both 10% - 50% and 50% - 90% relative to the mean control response.

An assessment of potential interaction between Cry1B.868 and Cry1Da_7 was evaluated with the concentration addition model. The concentration addition model is a well-established model to assess deviations from additivity and is based on the premise that the biological activity of the combined trait product can be predicted directly from the biological activity of the individual components. Under this model, a conclusion of additivity (i.e., no interaction) can be made if the predicted GI_{50} value calculated from the individual components is within the 95% CI of the observed GI_{50} value for the mixture (Levine et al.,2016; Tabashnik, 1992).

In seven-day diet incorporation bioassays with tissue from MON 95379, Cry1B.868-single, and Cry1Da_7-single, FAW demonstrated concentration-dependent responses for growth inhibition (Figure J-1). The observed GI_{50} values, from the combined analyses of three bioassay replicates,

for MON 95379, Cry1B.868-single and Cry1Da_7-single were 0.35 µg total protein (Cry1B.868 and Cry1Da_7)/ml diet, 0.34 µg Cry1B.868/ml diet, and 0.27 µg Cry1Da_7/ml diet, respectively (Table J-4).

Predicted and observed GI_{50} values for combined activity of Cry1B.868 and Cry1Da_7 were similar (Table J-5). The predicted GI_{50} value for Cry1B.868 and Cry1Da_7 using the concentration addition model was 0.33 µg total protein (Cry1B.868 and Cry1Da_7)/ml diet and was captured within the 95% CI for the observed GI_{50} value for MON 95379 (95% CI 0.29 to 0.42 µg total protein (Cry1B.868 and Cry1Da_7)/ml diet) indicating that combined activity was consistent with additivity. Additionally, the 95% confidence intervals for both the observed and predicted GI_{50} values for MON 95379 overlap with one another [95% CI 0.29 to 0.42 and 0.27 to 0.38 µg total protein (Cry1B.868 and Cry1Da_7)/ml diet)].

Table J-5. Observed seven-day FAW GI_{50} values and 95% confidence intervals (CI) for MON 95379, Cry1B.868-single, and Cry1Da_7-single and the predicted GI_{50} value and 95% CI for MON 95379.

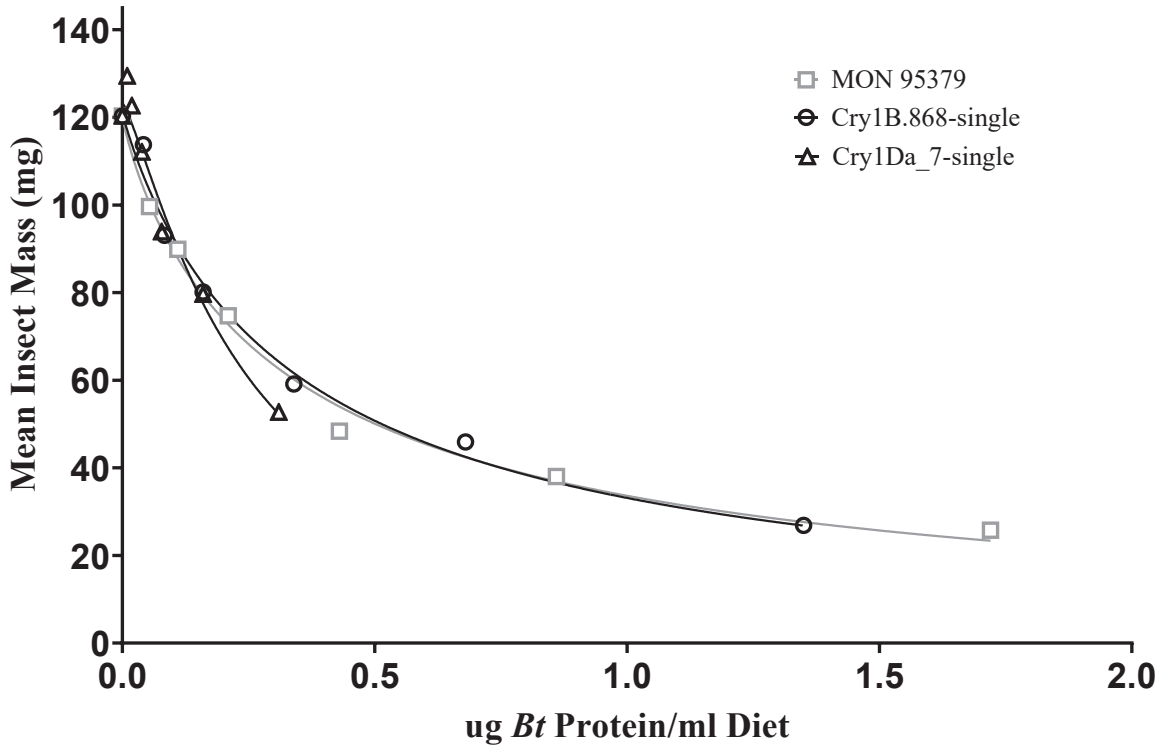
Substance Name (Treatment)	Estimated GI_{50} Value (µg *Bt* Protein/ml Diet)[1]	95% CI (µg *Bt* Protein/ml Diet)
MON 95379 (Observed)	0.35	0.29 – 0.42
Cry1B.868-single	0.34	0.28 – 0.40
Cry1Da_7-single	0.27	0.20 – 0.34
Substance Name (Treatment)	**Predicted GI_{50} Value (µg Protein/ml Diet)[1,2]**	**95% CI (µg *Bt* Protein/ml Diet)[3]**
MON 95379 (Predicted)	0.33	0.27 – 0.38

[1]The slope parameter for concentration response curves was shared for each regression since the slopes were determined to not be different for each treatment.

[2]Predicted GI_{50} value for MON 95379: 1/Predicted GI_{50} = 0.835/0.34 + 0.165/0.27

[3]Calculated using the delate method.

Figure J-2. Tissue from MON 95379, Cry1B.868-single, and Cry1Da_7-single resulted in concentration-dependent growth inhibition in seven-day diet-incorporation FAW bioassays (GraphPad, 2019).



Study Conclusions

These results demonstrate that the activity of Cry1B.868 and Cry1Da_7 is consistent with additivity (i.e., no interaction) in the combined trait product, MON 95379. Predicted and observed activity levels differed by only 6% (with overlapping 95% confidence intervals). The lack of interaction in addition to large margins of exposure and lack of adverse effects demonstrated in the NTO assessment support the conclusion that Cry1B.868 and Cry1Da_7 can be assessed independently.

J.1.4. Hazard Index Assessment for CryB.868 and Cry1Da_7

A supplementary assessment was conducted using the Hazard Index (HI) approach that conservatively evaluates the combined risk of Cry1B.868 and Cry1Da_7 to NTOs. Based on previous guidance and results from the interaction study, the expectation would be that MOE values established in the individual assessments of Cry1B.868 and Cry1Da_7 would be sufficiently protective and effects from the additive activity of Cry1B.868 and Cry1Da_7 would not be

anticipated. The HI represents a highly conservative approach to assess the potential impact of toxicant mixtures when their activities have been demonstrated to be additive (U.S. EPA, 2000). It should be noted that HI has been criticized in some contexts as overly conservative and should not be applied to substances below their no-effect levels because it could falsely identify risk where none exists. The HI approach is based on the concentration model and calculated as the summation of risk quotients (RQs):

$$Hazard\ Index = \sum_{i=1}^{n} \frac{Estimated\ Environmental\ Concentration}{No\ Mortality\ Level}$$

Adapting this approach for MON 95379 maize, the RQs of Cry1B.868 and Cry1Da_7 were calculated for each NTO as the estimated environmental concentrations (EECs) divided by the highest concentration tested for which there was no observed effect on survival. The survival endpoint was selected for this analysis because it was the endpoint that was common for all NTO studies. The RQs for each protein were summed to calculate the HI for each NTO and is shown in the Table J-6 below:

Table J-6. Calculated Risk Quotients (RQ) and Hazard Index (HI) Values for Cry1B.868 and Cry1Da_7 as Expressed in MON 95379 Maize

Test Organism	Protein	EEC (ppm)	No Mortality Level (ppm)	RQ	HI
Coleomegilla maculata	Cry1B.868	111.4	3500	0.0318	0.1712
	Cry1Da_7	20.9	150	0.1393	
Poecilus cupreus	Cry1B.868	111.4	3500	0.0318	0.0841
	Cry1Da_7	20.9	400	0.0523	
Geocoris punctipes	Cry1B.868	111.4	3500	0.0318	0.0736
	Cry1Da_7	20.9	500	0.0418	
Pediobius foveolatus	Cry1B.868	111.4	3500	0.0318	0.0736
	Cry1Da_7	20.9	500	0.0418	
Chrysoperla rufilabris (larva)	Cry1B.868	111.4	3500	0.0318	0.0736
	Cry1Da_7	20.9	500	0.0418	
Chrysoperla sp. (adult)	Cry1B.868	61.9	700	0.0884	0.0887
	Cry1Da_7	0.125	500	0.0003	
Folsomia candida	Cry1B.868	0.4	3500	0.0001	0.0005
	Cry1Da_7	0.2	500	0.0004	
Eisenia andrei	Cry1B.868	0.4	3500	0.0001	0.0005
	Cry1Da_7	0.2	500	0.0004	
Apis mellifera (adult)	Cry1B.868	61.9	900	0.0688	0.0690
	Cry1Da_7	0.125	500	0.0003	
Apis mellifera (larva)	Cry1B.868	61.9	900	0.0688	0.0690
	Cry1Da_7	0.125	500	0.0003	

USDA and EPA guidance states that only effects at 1× field expression levels environmental constitute risk (U.S. EPA, 2007). Based on this guidance, only HI values >1 would indicate potential risk from the additive activity of Cry1B.868 and Cry1Da_7. The HI values for nearly all tested species were <0.1 and thus, indicate minimal risk of additive activity to NTOs. The lone exception was *C. maculata* where the HI was >0.1 (but less than 1.0) due to the RQ for Cry1Da_7. The RQ for Cry1Da_7 was relatively higher because the test concentrations were adjusted to account for observed biological activity in test diet, but there was no adverse effect on survival of *C. maculata* exposed to Cry1Da_7. Applying the HI approach to Cry1B.868 and Cry1Da_7 is expected to be conservative because no adverse effects on survival were observed in Tier I testing resulting in unbounded no mortality levels (e.g., no lowest observed effect concentrations (LOECs) were determined). Thus, RQs calculated from test concentrations where effects on survival would actually occur (e.g., LOEC, LC_{50}, etc.) are likely quite smaller.

Conclusions

The study described above demonstrates that Cry1B.868 and Cry1Da_7 act independently, with additive activity in a species that is susceptible to both proteins. This result is consistent with published observations that Cry1B.868 and Cry1Da_7 act through distinct receptors (Wang et al., 2019). Since Cry1B.868 and Cry1Da_7 act independently and have high margins of exposure to NTOs, they can be assessed independently. Furthermore, the additive activities of Cry1B.868 and Cry1Da_7 as expressed in MON 95379 maize is not expected to pose a risk to NTOs.

J.2. Methods for Aerobic Soil Degradation of Cry1B.868 and Cry1Da_7 Protein from MON 95379 Maize

The purpose of this study was to estimate the aerobic degradation rate of the Cry1B.868 and Cry1Da_7 proteins derived from MON 95379 lyophilized maize leaf tissue in agricultural soils. The soils were exposed to Cry1B.868 and Cry1Da_7 through the incorporation of MON 95379 leaf tissue in soil. To serve as a negative control, conventional maize leaf tissue, which does not produce either Cry1B.868 or Cry1Da_7 protein, was incorporated into soil at the same rate as MON 95379 tissue. Three different soil types with diverse physicochemical characteristics were evaluated and were classified as sandy loam, silt loam and clay loam.

MON 95379 and conventional leaf tissues were incorporated into soils at the relative incorporation rate of 10-fold above those levels expected in the field. The amount of lyophilized leaf tissue to be incorporated into the soil was determined by calculating the amount of biomass present in a field that could enter the soil under standard cultivation conditions (1× field load) and is described in Section VII.B.

Vessels containing soil and incorporated maize tissue were incubated at an approximate temperature of 22 °C with soil moisture maintained at approximately 86% field moisture capacity. Samples were collected at 9 time points during a total incubation period of 213 days. Incubated

soil samples were extracted and analyzed for Cry1B.868 and Cry1Da_7 concentrations using enzyme-linked immunosorbent assays (ELISA) specific for each protein.

In addition, insecticidal activity of MON 95379 incorporated into soil was determined by a diet incorporation insect bioassay against a target lepidopteran species, fall armyworm (*Spodoptera frugiperda*). Fall armyworm (FAW) was selected as a test species because of its high sensitivity to the Cry1 class of proteins. Because the bioassay cannot distinguish activity from Cry1B.868 versus Cry1Da_7, the results were used qualitatively to evaluate temporal changes in activity and the soil concentrations from the ELISA analysis were used to calculate dissipation rate estimates.

The times to 50% dissipation (DT_{50}) and 90% dissipation (DT_{90}) for Cry1B.868 and Cry1Da_7 in each soil type were calculated from the ELISA results using a modified three-parameter logistic model and were applied at the sampling interval where maximum soil concentrations were observed (Section VII.B).

J.3. Evaluation of the potential effects of Cry1B.868 Protein on Larvae of the Ladybird Beetle, *Coleomegilla maculata*

Materials and Methods

Materials

Test material:	
Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	200 µg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment
Test organisms:	
Species:	*Coleomegilla maculata*
Age:	First instar, <24 hour old
Diet/Food:	Artificial diet provided *ad libitum*
Environmental conditions:	
Temperature:	27 ºC
Relative Humidity:	60%
Photoperiod:	14 hours light / 10 hours dark

Study Design and Methods

Experimental treatments: Cry1B.868 protein (in a buffer vehicle) was incorporated into an artificial agar-based diet at concentrations of 700 and 3500 µg Cry1B.868 protein/g diet. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate buffer solution, pH 11.5 incorporated into the diet at the same level as in the 3500 µg Cry1B.868 protein diet), and a positive control diet containing potassium arsenate at 200 µg/g diet were included in the study. Each treatment was incorporated into a diet medium and provided *ad libitum* and continuously to individually housed *C. maculata* larvae (target n = 30 per treatment) until the test larvae develop into adults. Freshly-defrosted aliquots of the appropriate treatment diet were provided in each test arena and renewed every 48 hours. Each test arena consisted of an inverted 60 mm × 15 mm Petri dish containing a filter paper, moistened with approximately two drops of deionized water, and a Post-it® flag (25.4 mm x 43.2 mm). Freshly-defrosted treatment diet

(approximately one 1/8 section of a 1 mL diet aliquot) was placed on Post-it® flag in each test arena and renewed every 48 to 72 hours. A targeted two additional drops of water were added to each arena daily. The other test arena contents (e.g. frass) were removed or replaced as needed. There were approximately equal numbers of larvae per treatment per day.

Observations: Following the initiation of the diet exposures, daily observations were made to record larval survival and development, and bw of the newly-emerged adults.

Statistical calculations: Fisher's Exact test was performed to compare the survival between each treatment with the buffer control. Treatment effects on the time of development to adult and adult body mass were analyzed with a linear model using SAS PROC Mixed. Pair-wise comparisons with the buffer control were defined within the linear model and tested using t-tests with significant difference at $\alpha = 0.05$.

Results

C. maculata larval survival was 90% in the negative control and 93% in the buffer control diet treatments, compared with 83.3% and 93.1% survival in the 700 and 3500 µg Cry1B.868 protein/g diet concentrations, respectively. There were no significant differences in survival between either test treatment level and the buffer control ($p>0.05$). The positive control treatment resulted in 100% mortality.

The mean development time was 21.4d in the negative control and 21.3d in the buffer control, compared with 20.8d and 21.6d in the 700 and 3500 µg Cry1B.868 protein/g diet concentraions, respectively. There were no significant differences in development time between either test treatment level and the buffer control ($p>0.05$). No insects developed into adults in the positive control treatment.

The mean adult weight was 10.0 mg in the negative control and 10.0 mg in the buffer control, compared with 10.0 mg and 9.2 mg in the 700 and 3500 µg Cry1B.868 protein/g diet concentraions, respectively. There was no significant difference in mean adult weight between the 700 µg Cry1B.868/g treatment level and the buffer control ($p>0.05$). There was a significant decrease in mean adult weight between the 3500 µg Cry1B.868/g, treatment level and the buffer control ($p = 0.046$).

Assay Validity Criteria

The survival of *C. maculata* larvae was 90% in the negative control and 93.3% in the buffer control, indicating there was no adverse effect from the buffer on survival. These results met acceptance criteria of having ≤20% mortality in the control. The positive control treatment resulted in 100% mortality demonstrating sensitivity of the test species.

The results of the diet-incorporation insect bioassay analysis demonstrate that the Cry1B.868 protein incorporated into the *C. maculata* diet had the expected level of biological activity, was homogenous through the test diet, was stable under test conditions and frozen storage.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous dietary exposure to Cry1B.868 protein at concentrations of either 700 or 3500 µg Cry1B.868 protein/g diet had no effects on either the survival or development time of the *C. maculata* larvae. There was also no effect on mean adult mass for *C. maculata* exposed to 700 µg Cry1B.868/g diet; however, at 3500 µg Cry1B.868/g diet, a statistically significant reduction (<10%) in mean mass was observed. The LC_{50} was >3500µg Cry1B.868 protein/g diet, the highest concentration tested, and the NOAEC was 700 µg Cry1B.868/g diet.

J.4. Evaluation of the Potential Effects of Cry1B.868 Protein on Larvae of the Carabid Beetle, *Poecilus cupreus*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	Teflubenzuron Pestanal (Sigma-Aldrich)
Dietery concentration:	0.032 mg per g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet at the same rate as for the 3500 µg/g test substance diet

Test organisms:

Species:	*Poecilus cupreus* larvae
Age:	1-2 d
Diet/Food:	Artificial diet provided *ad libitum*
Acclimation Period:	11 days after hatching

Environmental conditions:

Temperature:	18-19.8 ºC
Relative humidity:	64-83%
Photoperiod:	16 hours light / 8 hours dark
Illumination:	250 lux

Study Design and Methods

Experimental treatments: Cry1B.868 protein (in a buffer vehicle) was incorporated into a base diet mixture at two separate treatment concentrations, equivalent to 700 and 3500 µg Cry1B.868 protein/g diet. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate, pH 10.5 incorporated into the diet at the same rate as for the 3500 µg/g test substance diet), and a positive control of teflubenzuron at 32 µg/g diet to confirm the effectiveness of the dietary feeding were included in the study. The test arenas comprised lidded clear plastic tubes, each containing one beetle larva placed onto the surface of 25 g (dry weight) of a standard sandy soil moistened to 25% ± 5% of its maximum water-holding capacity (WHC). For the study, the soil-filled plastic tubes were stored upright in plastic boxes (one box per treatment), with lids containing 8 holes covered with nylon netting for ventillation and floor lined with capillary matting to raise the relative humidity around the tubes. Freshly-defrosted aliquots of appropriate treatment diets were provided daily to beetle larvae (n = 40 per treatment), starting with 24-48 hour old larvae, until all larvae had pupated or 21 days had passed.

Observations: The endpoints of the study were pre-imaginal mortality of the test insects, development time to adult emergence, and bw of the emerged adult beetles. The condition of visible beetle larvae was assessed three times per week for the first three weeks of the study, and twice each week thereafter until all larvae had pupated. Fresh bodyweight of adult beetles was recorded after the elytra had hardened. The study ended 45 DAT when it was determined that no more adults would emerge.

Statistical calculations: The overall percentage pre-imaginal mortality in each treatment was determined and the data corrected for any control deaths using Abbott's formula (Abbott, 1925). Multiple sequentially-rejective Fisher test after Bonferroni-Holm correction (one-sided, > control, $\alpha = 0.05$) was used to compare mortalities in the test substance and the buffer control treatment. The positive control treatment was compared to the buffer control by Fisher's exact binomial test (one-sided, > control, $\alpha = 0.05$). Adult bw was evaluated with a Dunnett's multiple t-test (one-sided, < control $\alpha = 0.05$) comparing test treatments to the buffer control. Effects on development time were evaluated with a Two-sample Mann Whitney *U*-test (one-sided, > control $\alpha = 0.05$) comparing test treatments to the buffer control.

Results

Pre-imaginal mortality was 12.5% in the negative control and 7.5% in the buffer control, compared with 22.5% and 12.5% mortality in the 700 and 3500 µg Cry1B.868 protein/g diet treatment concentrations, respectively. When adjusted for the deaths in the buffer control treatment, the test substance treatments resulted in 16.2% and 5.4% corrected mortality in the 700 and 3500 µg Cry1B.868 protein/g diet treatment concentrations, respectively. There were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$). No abnormal larvae, pupae or adults were seen, in any of the treatments. The positive

control treatment resulted in 100% pre-imaginal mortality (100% corrected) and this treatment differed significantly from the buffer control ($p<0.05$).

The mean emergence time for beetles surviving was 44.3 days in the negative control and 44.4 days in the buffer control, compared with 44.7 days and 44.6 days in the 700 and 3500 µg Cry1B.868 protein/g diet treatment concentrations, respectively. There was no significant difference in development time between either test treatment level and the buffer control ($p>0.05$).

The mean bw of individual beetles taken soon after their emergence was 63.5 mg in the negative control and 63.6 mg in the buffer control, compared to 61.2 mg and 62.8 mg in the 700 and 3500 µg Cry1B.868 protein/g diet treatment, respectively. There was no significant difference in adult bw between either test treatment level and the buffer control ($p>0.05$).

Assay Validity Criteria

The mortality of *P. cupreus* was 12.5% in the negative control and 7.5% in the buffer control, indicating there was no adverse effect from the buffer on survival. These results met assay acceptance criteria of having ≤20% mortality in the control. The positive control had 100% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated the Cry1B.868 test substance incorporated into the test treatment diets had the expected level of biological activity and was stable under test and frozen storage conditions. The homogeneity of the Cry1B.868 protein throughout the diet could not be confirmed, however, the concentrations detected in the test diet were never less than 3500 µg Cry1B.868 protein/g diet and, therefore, supports the conclusion that insects were exposed to the Cry1B.868 protein at the nominal test concentration.

Conclusion

Under laboratory test conditions, Cry1B.868 protein had no statistically significant effects on the survival of larvae, development or bw of the ground-dwelling carabid beetle, *Poecilus cupreus*, when provided orally at concentrations of 700 µg and 3500 µg Cry1B.868/g diet. The LC_{50} was >3500 µg Cry1B.868 protein/g diet and the NOAEC was 3500 µg Cry1B.868/g diet, the highest concentration tested.

J.5. Evaluation of the Potential Effects of Cry1B.868 Protein on Nymphs of the Big-Eyed Bug, *Geocoris punctipes*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	100 µg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment

Test organisms:

Species:	*Geocoris punctipes*
Age:	1 d
Diet/Food:	*Ephestia kuehniella* eggs and a slice of green bean pod provided *ad libitum*
Acclimation Period:	1 d

Environmental conditions:

Temperature:	25 ºC
Relative Humidity:	50%
Photoperiod:	14 hours light / 10 hours dark

Study Design and Methods

Experimental treatments: Cry1B.868 protein (in a buffer vehicle) was incorporated into a base diet mixture at two separate treatment rates, equivalent to 700 and 3500 µg Cry1B.868 protein/g diet each encapsulated to form a dome stored at -20°C. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate, pH 11.5 incorporated into the diet at the same rate as for the 3500 µg/g test substance diet), and a positive control diet containing potassium arsenate at 100 µg/g diet were included in the test. Two freshly-defrosted encapsulated diets (domes) of the appropriate treatment diet were provided in each test arena and renewed every 48 hours. The nymphs were allowed to feed *ad libitum* throughout the test duration. The nymphs after one-day acclimation were individually transferred into test arenas

(inverted Falcon petri dishes, 60×15mm, Corning, NY) and impartially assigned to each of the treatments by providing appropriate treatment diets. Each diet treatment consisted of a total of 30 nymphs individually housed in inverted petri dishes lined with a piece of filter paper on the bottom. One drop of distilled water was added on the filter paper every day at the time of observation to moisten the filter paper.

Observations: Daily observations were made to record test nymph survival, the time taken for the nymphs to develop into adulthood, and the fresh bw of the newly-emerged adults.

Statistical calculations: Fisher's Exact test was performed to compare the survival between predefined treatments. Treatment effects on the time of development to adult and adult body mass were analyzed with a linear model using SAS PROC Mixed. Pair-wise comparisons with the buffer control were defined within the linear model and tested using t-tests with significant difference at $\alpha = 0.05$.

Results

G. punctipes nymph survival was 86.7% in the negative control and 90% in the buffer control diet treatments, compared with 92.9% and 100% survival in the 700 and 3500 µg Cry1B.868 protein/g diet treatment concentrations, respectively. There were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$). The positive control treatment resulted in 100% mortality.

All surviving nymphs completed the development into adulthood. The mean nymph development time to adults was 31.0 days in the negative control and 29.4 days in the buffer control, compared with 30.3 days and 30.0 days in the 700 and 3500 µg Cry1B.868 protein/g diet treatment concentrations, respectively. There was a significant difference between the 700 µg Cry1B.868/g diet treatment level and the buffer control ($p = 0.0288$). However, there was no significant difference btween the 3500 µg Cry1B.868/g diet treatment level and the buffer control ($p = 0.1281$).

The mean bw of newly-emerged adults was 4.4 mg in the negative control and 5.1 mg in the buffer control, compared to 4.7 mg in both Cry1B.868 treatment levels. There were no significant differences in bw between either test treatment level and the buffer control ($p>0.05$).

Assay Validity Criteria

G. punctipes nymph survival was 86.7% in the negative control and 90% in the buffer control diet treatments, indicating there was no adverse effect from the buffer on survival. These results met assay acceptance criteria of having ≥80% survival The positive control treatment resulted in 100% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated that the Cry1B.868 protein in the treatment diet had the expected level of biological activity and was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous dietary exposure to Cry1B.868 protein at 3500 µg Cry1B.868 protein/g diet had no effects on the survival, growth, or development of the *G. punctipes* nymphs. The LC_{50} was >3500 µg Cry1B.868 protein/g diet and the NOAEC was 3500 µg Cry1B.868 protein/g diet, the highest concentration tested.

J.6. Evaluation of the Potential Effects of Cry1B.868 Protein on Adults of the Parasitoid Wasp, *Pediobius foveolatus*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	200 µg/ml diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment

Test organisms:

Species:	*Pediobius foveolatus*
Age:	1 d
Diet/Food:	30% honey/water (v/v) solution
Acclimation Period:	24 h

Environmental conditions:

Temperature:	25 ºC
Relative Humidity:	70%
Photoperiod:	14 hours light / 10 hours dark

Study Design and Methods

Experimental treatments: Cry1B.868 protein (in a buffer vehicle) was incorporated into 30% honey/purified water solution at two nominal concentrations of 700 and 3500 µg/ml 30% honey/purified water solution, respectively. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate, pH 10.5 incorporated into the diet at the same rate as for the 3500 µg/ml test substance diet), and a positive control diet containing potassium arsenate at 200 µg/ml diet were included in the study. The newly emerged *P. foveolatus* adults produced in laboratory from the parasitized *E. varivestis* larvae were supplied with 30% honey/water (v/v) solution and were impartially transferred into 162 cm^2 cell culture flasks. After approximately 24 hours of acclimation, 15 adults in each flask were impartially assigned to the five treatments and provided with appropriate treatment diet in two screened feeding dishes, replaced every two days.

Each treatment included four replicates. The adult wasps were allowed to feed *ad libitum* throughout the 21 days feeding period.

Observations: All wasps in all treatments were observed for mortality/survival every two days at the time of diet replacement. Dead wasps, if any, were removed from the test arenas and recorded.

Statistical calculations: Statistical analyses were not performed since no mortality was recorded in either Cry1B.868 treatment level as well as the negative and buffer controls and 100% mortality was recorded in positive control treatment.

Results

P. foveolatus adult survival was 100% in both 700 and 3500 µg Cry1B.868 protein/ml diet treatment levels as well as the negative and buffer controls. The positive control treatment resulted in 100% mortality (0% survival).

Assay Validity Criteria

The survival of *P. foveolatus* larvae in both the negative (water) and buffer control groups was 100%, indicating there was no adverse effect from the buffer on survival, and met the assay acceptance criteria of having ≥80% survival in the control. The positive control treatment resulted in 100% mortality demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated that the Cry1B.868 protein in the treatment diet had the expected level of biological activity and was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous dietary exposure to Cry1B.868 protein at concentrations of either 700 or 3500 µg Cry1B.868 protein/ml diet for 21 days had no effects on the survival of the *P. foveolatus* adults. The LC_{50} was >3500 µg Cry1B.868 protein/ml diet, the highest concentration tested.

J.7. Evaluation of the Potential Effects of Cry1B.868 Protein on Adults of the Green Lacewing, *Chrysoperla carnea*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	dimethoate
Dietery concentration:	0.04 µg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet at the same rate as for the 3500 µg/g test substance diet

Test organisms:

Species:	*Chrysoperla carnea*
Age:	24 h
Sex:	10 males/10 females per treatment group
Diet/Food:	None

Environmental conditions:

Temperature:	23.9-25.6 ºC
Relative humidity:	67-76%
Photoperiod:	16 hours light / 8 hours dark
Illumination:	3100-4100 lux

Study Design and Methods

Experimental treatments: *Chrysoperla carnea* adults, less than 24 h old, were exposed to five treatments, Cry1B.868 protein at 3500 µg protein/g diet, Cry1B.868 µg protein at 700 protein/g diet, a negative control (purified water only), buffer control consisting of test substance buffer (25 mM sodium carbonate, pH 11.5) at a volume equivalent to the highest test substance concentration, and a positive control of dimethoate at 0.04 µg/g diet to confirm the effectiveness of the dietary feeding. The test arenas comprised clear polystyrene boxes with close-fitting lids. The dishes of the treated diet, and dishes of honey-water and water-only, were placed on the floor of each box. For each treatment there were two replicate boxes, each containing 20 adult lacewings (i.e. 40 per treatment) with 10 female and 10 male lacewings impartially assigned to each test arena except positive control treatment arenas that receievd 11 female and 9 male lacewings. Any insects

that appeared to have been damaged during the transfer process were replaced immediately. Freshly-defrosted aliquots of appropriate treatment diets were provided to newly-emerged adult lacewings three times per week, for two weeks.

Observations: The endpoint of the study was an assessment of the adult mortality.. The adults were assessed every 1-3 days for 14 days and finally at 14 days after treatment, from which the overall percentage mortality in each treatment was calculated.

Statistical calculations: The percentage adult mortality in each treatment was determined and the data corrected for any insect deaths in the buffer control using Abbott's formula (Abbott, 1925). Multiple sequentially-rejective Fisher test after Bonferroni-Holm correction (one-sided, > control, $\alpha = 0.05$) was used to compare mortalities in the test substance and the buffer control treatment. The positive control treatment was compared to the buffer control by Fisher's exact binomial test (one-sided, > control, $\alpha = 0.05$).

Results

Mortality was 15% in the negative control and 15% in the buffer control, compared with 40% and 35% mortality in the 700 µg and 3500 µg Cry1B.868 protein/g diet treatment concentrations, respectively. There was a significant increase in mortality for both Cry1B.868 treatment levels compared to the buffer control ($p<0.05$). The positive control treatment resulted in 62.5% mortality (55.9% corrected) and this treatment differed significantly from the buffer control ($p<0.05$).

Assay Validity Criteria

Mortality was 15% in the negative control and 15% in the buffer control, indicating the was no adverse effect from the buffer on survival. These results met assay acceptance criteria of ≤20% mortality in the control. The positive control had 62.5% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis by sensitive insect bioassay demonstrated that the Cry1B.868 protein incorporated into diet had the expected level of biological activity, was considered homogenous and was stable under test and frozen conditions.

Conclusion

Under laboratory test conditions, continuous dietary exposure of Cry1B.868 protein to adults of the green lacewing, *C. carnea*, for 14 days resulted in statistically significant reduced survival at both the 700 and 3500 µg protein/g diet concentrations. The LC_{50} was >3500 µg Cry1B.868 protein/g diet and the NOAEC could not be determined in the study.

J.8. Evaluation of the potential effects of Cry1B.868 Protein on Larvae of the Green Lacewing, *Chrysoperla rufilabris*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	0.1 mg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet at the same rate as for the 3500 mg/kg test substance diet

Test organisms:

Species:	*Chrysoperla rufilabris*
Age:	Second instar larvae
Diet/Food:	Untreated diet and water soaked cotton balls
Acclimation Period:	24 hours

Environmental conditions:

Temperature:	24-26ºC
Relative humidity:	52-69%
Photoperiod:	16 hours light / 8 hours dark
Illumination:	550-710 lux

Study Design and Methods

Experimental treatments: Second instar *Chrysoperla rufilabris* larvae (3-5 days from egg hatch) were exposed to six treatments, Cry1B.868 protein at 110 µg protein/g diet, Cry1B.868 protein at 700 µg protein/g diet, Cry1B.868 protein at 3500 µg protein/g diet, a negative control consisting of meat-based diet without any test substance or buffer added, buffer control consisting of test substance buffer (25 mM sodium carbonate buffer solution, pH 11.5) at a volume equivalent to the highest test substance concentration, and a positive control of potassium arsenate at 0.1 mg/g diet to confirm the effectiveness of the dietary feeding for 24 days. The test vessels comprised Petri dishes with lids. Lacewing larvae were acclimated for 24 hours prior to test initiation and were impartially allocated to the test vessels. Each treatment and control had 40 replicate test vessels with each vessel containing one test organism. Individual vessels were randomly assigned to the

treatments using computer generated random numbers. Test vessels were then randomly placed within the environmental chamber during the exposure. At study initiation, one freshly-defrosted 50 µL pellet of the appropriate control or treatment level was added to each test vessel along with one moistened small cotton ball with both being replaced at every 48-hour interval until organisms reached pupal state, when organisms do not actively feed on diet. The study was terminated on day 24 and pupae that had not emerged were considered dead.

Observations: The endpoints of the study were percentage pre-imaginal survival, development time from larvae to emerged adult, and adult weight at emergence. The health of the organisms was observed and recorded daily. Pupation and adult emergence were recorded when observed. When adult emergence was observed, the replicate was removed from the study and placed in a freezer for euthanization.

Statistical calculations: The treatment data were tested for normality and homogeneity of variance using the appropriate qualifying test. Fisher's exact test with Bonferroni-Holm correction was used to compare survival between the treatment and negative control groups. Effects on development time were evaluated with a Dunnett's multiple comparison test comparing test treatments to the negative control. Adult bw was evaluated with a Jonckheere-Terpstra's Step-down test comparing the test treatments and the negative control. All comparisons were made at ≥95% level of certainty ($\alpha = 0.05$) and compared on a per replicate basis.

Results

The mean pre-imaginal survival was 80% in the negative control and 78% in the negative control, compared with 70, 73, and 75% in the 110, 700, and 3500 mg/kg treatment levels, respectively. There were no significant reductions in pre-imaginal survival between any test treatment level and the negative control ($p>0.05$).

The mean weight for adults at emergence was 0.0079 g in the negative control and 0.0073 g in the buffer control, compared with 0.0076 g, 0.0074 g, and 0.0074 g in the 110, 700, and 3500 mg/kg treatment levels, respectively. There were no significant reductions in adult body weight between any test treatment level and the negative control ($p>0.05$).

The mean development time from larvae to emerged adult was 19 days in the negative control and 20 days in the buffer control, compared with 20 days for all Cry1B.868 test treatment levels. There were no significant reductions in development time between the test treatment levels and the negative control ($p>0.05$).

Assay Validity Criteria

Larval mortality was 8% in the negative control and 3% in the buffer control, indicating there was no adverse effect from the buffer on survival. These results met assay acceptance criteria of ≤20% mortality in the control. The positive control had 100% larval mortality, demonstrating sensitivity of the test species.

The results of the dose confirmation demonstrated that the Cry1B.868 protein incorporated into diet had the expected level of biological activity, was homogeneous throughout the test diet, and was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, Cry1B.868 protein had no statistically significant effects on the survival, development of larvae, and adult weight of the the green lacewing, *Chrysoperla rufilabris* at concentrations of 110 µg, 700 µg, and 3500 µg Cry1B.868/g diet fed continuously during their development for 24 days. The LC_{50} was >3500 µg Cry1B.868 protein/g diet and the NOAEC was 3500 µg Cry1B.868/g diet, the highest concentration tested.

J.9. Evaluation of the Potential Effects of Cry1B.868 Protein on Adults of the Green Lacewing, *Chrysoperla rufilabris*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	500 µg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet at the same rate as for the test substance diet

Test organisms:

Species:	*Chrysoperla rufilabris*
Age:	< 48 h
Diet/Food:	40% honey/water (v/v) solution provided *ad libitum*
Acclimation period	24 h

Environmental conditions:

Temperature:	25 ºC
Relative humidity:	75%
Photoperiod:	16 hours light / 8 hours dark

Study Design and Methods

Experimental treatments: *Chrysoperla rufilabris* adults, less than 48 h old, were exposed to six treatments, Cry1B.868 protein at 110 µg protein/g diet, Cry1B.868 µg protein at 350 protein/g diet, Cry1B.868 µg protein at 700 protein/g diet, a negative control diet consisting of purified water only, buffer control consisting of test substance buffer (25 mM sodium carbonate, pH 11.5) at a volume equivalent to the highest test substance concentration, and a positive control of potassium arsenate at 500 µg/g diet to confirm the effectiveness of the dietary feeding. Each treatment was replicated three times with targeted 15 adults per replicate. The test arenas comprised 10.8-cup plastic food storage container with two feeding stations and the treatment diets were replaced every 48 hours. The newly emerged adults (< 48 h) in each test arena were provided with 40% honey/water (v/v) solution in two feeding stations and acclimated for 24 hours prior to inclusion in feeding test. The adults in each replicate allowed to feed *ad libitum* on the treatment diet for 14

days. Freshly-defrosted aliquots of treatment diet were provided to adult lacewings on seven occasions over a 14-day period in two feeding stations placed in each replicate test arena.

Observations: The endpoint of the bioassay was a daily assessment of the adult mortality for 14 days, from which the percentage mortality in each treatment was calculated.

Statistical calculations: SAS PROC Freq was used to conduct the Fisher's exact test to compare survival rate between treatments at significance $\alpha = 0.05$. SAS PROC MEANS was used to calculate the survival (%) across replicates for each treatment.

Results

Survival of *C. rufilabris* adults was 97.8% in the negative control and 95.6% in the buffer control, compared with 91.1%, 95.7% and 100.0% survival in the 110, 350 and 700 µg Cry1B.868 protein /g diet treatment concentrations, respectively. There were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$). The positive control treatment resulted in 100% mortality.

Assay Validity Criteria

Survival of *C. rufilabris* adults was 97.8% in the negative control and 95.6% in the buffer control, indicating there was no adverse effect from the buffer on survival. These results met assay acceptance criteria in having ≥80% survival in the control. The positive control treatment resulted in 100% mortality, demonstrating sensitivity of the test species.

The results of diet analysis indicated that the Cry1B.868 protein in diet samples had the expected level of biological activity, was homogeneous in diet, and was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, continuous dietary exposure of Cry1B.868 protein to adults of the green lacewing, *C. rufilabris* for 14 days resulted in no significant effect on survival at 110, 350 and 700 µg protein/g diet treatments. The LC_{50} was >700 µg Cry1B.868 protein/g diet, the highest concentration tested.

J.10. Evaluation of the Potential Effects of Cry1B.868 Protein on the Springtail, *Folsomia candida*

Materials and Methods

Materials

Test material:	
Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Reference and/or positive control:	Teflubenzuron
Dietery concentration:	0.2 mg per g diet (see: Study plan deviations)
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment
Test organisms:	
Species:	*Folsomia candida*
Age:	12 d
Diet/Food:	Artificial diet provided *ad libitum*
Acclimation Period:	11 days after hatching
Environmental conditions:	
Temperature:	19.3-20.8 ºC
Photoperiod:	16 hours light / 8 hours dark
Illumination:	600-780 lux

Study Design and Methods

Experimental treatments: Cry1B.868 protein (in a buffer vehicle) was incorporated into a diet medium of inactivated yeast at two separate treatment concentrations, equivalent to 3500 and 700 µg Cry1B.868 protein/g diet. For the study, these two dietary treatments were compared to a buffer control diet to which 25 mM sodium carbonate buffer solution (pH 11.5) was added at an equivalent concentration as in the Cry1B.868 protein treatments. A negative control diet (purified water only) and a positive control diet treated with a technical-grade sample of teflubenzuron, at a rate equivalent to 0.2 mg/g diet were included in the study. Freshly-defrosted aliquots of appropriate treatment diets were provided continuously to batches of confined springtails over a 28-day period. The test arenas comprised lidded jars, the bases of which were lined with a plaster-of-Paris and charcoal substrate. Ten juvenile springtails (12 days old) were placed in each jar (n =

4 per treatment) and were fed ad libitum throughout the study. Freshly-treated aliquots of appropriate treatment diet were provided on the day of study initiation (Day 0) and additional freshly-defrosted aliquots were then provided every 2-3 days.

Observations: For the study, the numbers both of surviving adults and F1 progeny in each replicate arena were recorded at 28 days after treatment (DAT). It was assumed that any adult springtails that were recovered would have been those originally introduced and that any shortfall in the original number was an indication that they had died during the study. The mean number of juveniles produced at 28 DAT, and the standard deviation and the coefficient of variation were calculated from the replicates in each treatment. The numbers of surviving springtails were used to calculate the percentage mortality of the springtails originally introduced in each treatment.

Statistical calculations: The 28-day mortality data for the individual test substance treatments were compared to those for the buffer control using multiple sequentially-rejective Fisher test after Bonferroni-Holm correction (one-sided greater, $\alpha = 0.05$). Following a check for normal distribution of the data (Shapiro-Wilk test, $\alpha = 0.05$) and for equality of variances (Levene's test, $\alpha = 0.05$), test treatments were compared to the buffer control by multiple sequentially-rejective t-test after Bonferroni-Holm correction (one-sided smaller, $\alpha = 0.05$). The positive control treatment was compared to the buffer control by Student t-test for homogeneous variances (one-sided smaller, $\alpha = 0.05$).

Results

At 28 days, there was 5% mortality in both the negative and buffer control diet treatments, compared with 5% mortality in both the 700 and 3500 µg Cry1B.868 protein/g diet treatment concentrations. When adjusted for the deaths in the buffer control treatment, both the test substance treatments resulted in 0% corrected mortality. There was no significant difference in mortality between either test treatment level and the buffer control ($p>0.05$ for both concentrations). The positive control treatment resulted in 93% mortality (92% corrected) and this treatment therefore differed significantly from the buffer control ($p<0.05$).

The mean number of progeny produced per treatment was 331 in the negative control and 358 in the buffer control, compared with 330 and 304 in the 700 and 3500 µg Cry1B.868 protein/g diet treatment concentrations, respectively. There were no significant differences in reproduction between either test treatment level and the buffer control ($p>0.05$).

Assay Validity Criteria

The mortality of *F. candida* was 5% in both the negative and buffer controls, indicating there was no adverse effect from the buffer on survival. These results met the assay acceptance criteria of having ≤20% mortality in the control. The mean number of juveniles recorded in the negative control treatment was at least 100 per replicate at the end of the study and the coefficient of variation of reproduction in the negative control was ≤30% meeting acceptance criteria. The positive control treatment resulted in 93% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated that the test substance, Cry1B.868 protein in test diet had the expected level of biological activity, was homogenous throughout the freshly-prepared test diet and was stabile under test and storage conditions.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous exposure to Cry1B.868 protein for 28 days at concentrations of either 700 or 3500 µg Cry1B.868 protein/g diet had no effects on either the survival or reproduction rate of the springtail *Folsomia candida*. The LC_{50} was >3500 µg Cry1B.868 protein/g diet and the NOAEC was 3500 µg Cry1B.868 protein/g diet, the highest concentration tested.

J.11. Evaluation of the Potential Effects of Cry1B.868 Protein on the Earthworm, *Eisenia andrei*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 protein
Description:	Clear dark amber solution
Purity corrected concentration:	28.7 mg/mL
Vehicle and/or positive control:	2-chloroacetamide (tested in a separate study)
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the soil to achieve the same concentration of buffer per g soil as used for the test substance treatments

Test organisms:

Species:	*Eisenia andrei*
Age:	~8 months
Diet/Food:	None
Acclimation Period:	1 day

Environmental conditions:

Temperature:	20.2-22.0 ºC
Photoperiod:	24 hours light
Illumination:	490-550 lux

Study Design and Methods

Experimental treatments: The Cry1B.868 protein held in a buffer solution was incorporated into an artificial soil medium at two separate treatment concentrations, equivalent to 3500 and 700 µg Cry1B.868 protein/g soil dry weight. For the study, these two treatments were compared to a control soil treated with either 25 mM sodium carbonate buffer solution, pH 11.5 at the same concentration as used for the test substance treatments (buffer control) or with purified water (negative control). To confirm sensitivity of the test species, a positive control treatment of 2-chloroacetamide was evaluated in a separate, GLP-compliant study within 2 months of the study. The artificial soil substrate (containing 10% w/w peat), was moistened to 50% of its pre-determined maximum water-holding capacity at the time of treatment. The treated soil was held within 1-L-capacity lidded jars (n = 4 per treatment) and 10 adult *E. andrei* (approx. 8 months old, with an individual fresh weight of 358-598 mg and with a visible clitellum) were weighed and

introduced into each jar immediately after treatment application. Each treatment was replicated four times for a total of 40 earthworms exposed per treatment. No food was provided throughout the duration of the study.

Observations: For the study, the numbers of surviving adult earthworms in each test arena were recorded at 7 and 14 DAT. It was assumed that any adult earthworms that were recovered would have been those originally introduced and that any shortfall in the original number was an indication that they had died during the study. The mean percentage change in weight of the earthworms in the controls and test-substance treatments over 14 days was calculated. The earthworms were gently cleaned using purified water and individually re-weighed at 14 DAT after removing excess moisture to determine the mean weight of individuals in each replicate. The percentage change in mean weight per replicate was derived by comparing the mean weight recorded at 0 DAT with that measured again at 14 DAT for earthworms in each replicate. The earthworms were also examined for any other harmful effects (e.g. behavioral abnormalities or open wounds) at 0, 7 and 14 DAT.

Statistical calculations: Since there was no mortality in the controls, it was unnecessary to correct for mean control mortality using Abbott's formula. Fisher's Exact binomial test with Bonferroni correction (one-sided, > control, $\alpha = 0.05$) was used to compare mortalities in the individual test treatments with the buffer control treatment. Following a check for normal distribution of the data (Shapiro-Wilk test, $\alpha = 0.05$) and for equality of variances (Levene's test, $\alpha = 0.05$), test treatments were compared to the buffer control by multiple sequentially-rejective t-test after Bonferroni-Holm correction (one-sided smaller, $\alpha = 0.05$).

Results

At 14 days, there was no mortality in both the negative and buffer control treatments, compared with 3% and 0% mortality in 700 and 3500 µg Cry1B.868 protein/g soil dry weight treatment concentrations, respectively. There were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$).

The mean percentage change in earthworm weight per treatment was equivalent to a decrease of 7.9% in the negative control and 4.4% in the buffer control, compared with an increase of 4.4% and an increase of 43.3% in the 700 and 3500 µg Cry1B.868 protein/g soil dry weight treatment concentrations, respectively. There was not a significant decrease in bw between either test treatment level and the buffer control ($p>0.05$). At both 7 and 14 DAT, all of the earthworms in all of the treatments appeared healthy and active.

Assay Validity Criteria

The mortality of *Eisenia andrea* was 0% in the negative control and 0% in the buffer control group, indicating there was no adverse effect of the buffer on survival. These results met the assay

acceptance criteria of ≤10% mortality in the control. The mean post-treatment weight of the negative control group decreased by <20% compared to the start of the study, meeting acceptance criteria.

As this was an acute study with a single application of the Cry1B.868 protein, no direct measurement was made of test substance homogeneity in the soil. However, care was taken to mix the treated soil thoroughly. No measurement was made of the stability of the test substance in the soil, but the test earthworms were placed onto the soil immediately after the treatment of each arena.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous exposure to Cry1B.868 protein for 14 days in an artificial soil containing 10% w/w peat at concentrations of either 700 or 3500 µg Cry1B.868 protein/g soil dry weight had no significant treatment effects on the survival or biomass of the earthworm *Eisenia andrei*. Additionally, no abnormal behaviors were observed in any of the treatments. The LC_{50} was >3500 µg Cry1B.868 protein/g soil dry weight and the NOAEC was 3500 µg Cry1B.868 protein/g soil dry weight, the highest concentration tested.

J.12. Evaluation of the Potential Effects of Cry1B.868 Protein on Adults of the Honey Bee, *Apis mellifera*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868
Description:	Liquid
Purity corrected concentration:	28.7 mg/mL
Positive control:	Dimethoate
Dietery concentration:	0.65 mg/kg diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 11.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment

Test organisms:

Species:	*Apis mellifera* L.
Age:	≤2d at dosing
Diet/Food:	50% sucrose/purified water solution provided *ad libitum*
Acclimation Period:	1 d

Environmental conditions:

Temperature:	33°C
Relative Humidity:	50-70 %
Photoperiod:	Maintained in the dark thoughout duration of test

Study Design and Methods

Experimental treatments: Honey bee adults, ≤2 days old, were exposed to five treatments, Cry1B.868 protein at 180 µg/g in combination with 50% sucrose/purified water solution, Cry1B.868 protein at 900 µg/g in combination with 50% sucrose/purified water solution, a negative control consisting of 50% sucrose/purified water solution only, buffer control consisting of test substance buffer (25 mM sodium carbonate buffer solution, pH 11.5) at a volume equivalent to the highest test substance concentration, and a positive control of dimethoate at 0.65 mg/kg to confirm the effectiveness of the dietary feeding. Five cages (replicates) for each five treatments comprised a total of 25 cages. Individual cages contained 10 adult bees obtained from a local hive mainatined by Eurofins that was apparently healthy, queen-right, and had not been treated for

control of Varroa or pathogens within four weeks of the study. Once the honey bee adults were caged impartially, each cage was assigned to a treatment/replicate in a non-systematic fashion. After all adult bees were acclimated under red light with 50% (w/v) sucrose solution provided *ad libitum*, each cage was provided with the appropriate treatment diet solution through two feeders (syringes, each containing ~2.5 mL diet) inserted through the lid of the test chamber and bees were allowed to feed *ad libitum*. Feeders were replaced daily and were weighed prior to placing them into the cages and again after removal 24 ± 2 hours later to measure the amount of diet consumed in each replicate. The study duration was 10 days with adult bees being observed daily for mortality, behavioral and toxicological responses.

Observations: All treatments were observed for mortality, behavioral and toxicological responses once within the first four hours after initial treatment and at approximately 24-hour intervals (from dosing) thereafter. Abnormal behavior was determined by comparing honey bees in the treatment groups with those in the negative control group. Dead honey bee adults remained in their respective cages until study completion to avoid unnecessary disturbances. Consumption per bee in each replicate was calculated for each day using the consumption data adjusted for evaporative loss and the total number of bees alive in each replicate at the start of each feeding period (day). The consumption per bee values for all the replicates within a treatment level were then averaged for each day. Replicates were no longer included in the average consumption calculations after reaching 100% mortality.

Statistical calculations: The NOEC value was empirically determined by visual observation of the mortality data because mortality in the highest level was lower than in the negative control.

Results

Mortality of *Apis mellifera* adults was 12% in the negative control and 6% in the buffer control, compared to 22% and 8% mortality in the 180 µg Cry1B.868 protein/g and 900 µg Cry1B.868 protein/g groups, respectively. All surviving bees appeared normal at the end of the study. Positive control group mean mortality was 100%.

Average daily consumption over the 10-day exposure period were 28, 27, 30, 27 and 13 mg diet/bee/day in the negative control, 180 µg Cry1B.868 protein/g, 900 µg Cry1B.868 protein/g, buffer control and positive control groups, respectively. These results demonstrate that diet consumption was comparable in all groups, except positive control. The daily doses were calculated based on the average daily consumption values and were 4.89 and 26.6 µg Cry1B.868 protein/bee/day during the study for the 180 µg Cry1B.868 protein/g and 900 µg Cry1B.868 protein/g treatment levels, respectively.

Assay Validity Criteria

Mortality of *Apis mellifera* adults was 12% in the negative control and 6% in the buffer control, indicating there was no adverse effect from the buffer on survival. The positive control had 100% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated the Cry1B.868 test substance incorporated into the test treatment diets had the expected level of biological activity, was stable for 1 day under test condtions, 4 days at 4 ℃, and under frozen storage conditions.

Conclusion

Following oral exposure of adult honey bees under laboratory test conditions to Cry1B.868 protein for 10 days, the LC_{50} was >900 µg Cry1B.868 protein/g diet and the NOAEC was 900 µg Cry1B.868 protein/g, the highest concentration tested.

J.13. Evaluation of the Potential Effects of Cry1B.868 Protein on Larvae of the Honey Bee, *Apis mellifera*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868
Description:	Liquid
Purity corrected concentration:	28.7 mg/mL
Positive control:	Dimethoate
Dietery concentration:	7.42 µg/bee

Test organisms:

Species:	*Apis mellifera* L.
Age:	~6 d at dosing
Diet/Food:	Artificial diet (OECD 239)
Acclimation Period:	2 d after grafting

Environmental conditions:

Average Temperature:	34.47°C (Larval exposure phase)
	34.27°C (Pupal transfer to emergence)
Average Relative Humidity:	92.88 % (Larval exposure phase)
	76.80 % (Pupal transfer to emergence)
Photoperiod:	Maintained in the dark thoughout duration of study

Study Design and Methods

Experimental treatments: First instar larvae from three hives were transferred (grafted) to well plates containing untreated artificial diet and held in an incubator. Starting at two days after grafting, artificial diets containing the test or control substance were provided to larvae. Test diets were provided for a total of four days. Larvae were then held for up to 14 days after the completion of dosing in order to allow emergence of adult bees. Negative control (untreated artificial diet), buffer control (25 mM sodium carbonate buffer solution, pH 11.5 at a volume equivalent to the highest test substance concentration) and positive control (dimethoate, 7.42 µg/bee) groups were maintained concurrently. Each treatment and control group contained 16 larvae from each of the three hives, for a total of 48 larvae per treatment group. Nominal test levels were 180 and 900 µg Cry1B.868/g diet.

Observations: Starting on the first day of dosing (Day 3), larvae were observed daily until either mortality or adult emergence occurred. Cell cups containing dead larvae were removed from well plates after mortality was recorded. Observations of sublethal effects, including the presence of uneaten diet and larvae with reduced body size, were recorded on Days 7 and 8. Emerged bees from each replicate were checked to evaluate sublethal effects on morphology of emerged bees. By Day 20, approximately 14 days after final dosing, all bees had either emerged or died.

Statistical calculations: Dunnett's multiple comparison test was used to compare mortalities in the individual test treatments to the negative control. Mean larval survival and pupal survival were calculated but not be analyzed statistically. Sublethal effects were reported but not statistically analyzed.

Results

Mean mortality was 10% in the negative control 21% in the buffer control and no significant difference was detected according to t-test, ($p>0.05$). Mean mortality values in the 180 and 900 µg Cry1B.868 protein/g treatment groups were 17 and 21%, respectively. There were no significant differences in mortality between the test treatments and the negative control ($p>0.05$). At the end of the study all adult bees appeared healthy with no morphological deformities observed, except one bee in the buffer control.

Assay Validity Criteria

Cumulative larval mortality was less than 15% across replicates, adult emergence was higher than 70% on Day 20 in the negative control group, and larval mortality was higher than 50% by day 8 in the positive control group, meeting acceptance criteria.

The results of the diet analysis demonstrated the Cry1B.868 test substance incorporated in diets had the expected level of biological activity, was homogeneous throughout the diet, and was stable under test and frozen storage conditions.

Conclusion

Following dietary exposure of larval honey bees under laboratory test conditions to Cry1B.868 protein, the LC_{50} was >900 µg Cry1B.868 protein/g diet and the NOAEC for larval honey bees was 900 µg Cry1B.868 protein/g, the highest concentration tested.

J.14. Evaluation of the Potential Effects of Cry1B.868 Protein on the Northern Bobwhite Quail, *Colinus virginianus*

Materials and Methods

Materials

Test material:

Test item:	Cry1B.868 lyophilized powder
Description:	Solid
Purity corrected concentration:	0.74 mg/mg lyophilized powder
Control:	Bovine serum albumin (BSA) lyophilized powder

Test organisms:

Species:	Northern bobwhite (*Colinus virginianus*)
Age:	27 weeks
Sex:	5 males/5 females per treatment group
Weight:	189-223 g
Diet/Food:	Cargill game bird ration, *ad libitum*
Acclimation Period:	8 weeks

Environmental conditions:

Temperature:	23.9°C (Range: 22.4-25.6°C)
Humidity:	62% (Range: 37-75%)
Photoperiod:	8 hours light / 16 hours dark
Illumination:	625 lux

Study Design and Methods

Experimental treatments: After an acclimation period of 8 weeks, ten adult northern bobwhite were indiscriminately assigned to the treatment and control groups. The test dose substance (TDS) and control dose substance (CDS) was dosed using capsules at a rate of 3500 mg product/kg of bw. Nominal dosages used in this study were 0 mg/kg, 3500 mg of CDS/kg and 3500 mg TDS/kg. The birds were fasted for approximately 16.5 hours prior to dosing and individually weighed and dosed on the basis of mg of a.i/kg of bw. At the experimental start, two capsules containing either

the CDS or TDS was orally inserted into the crop of each bird. A third capsule was dosed after approximately four hours following the initial dosing. The negative control birds received three empty capsules of the same size used for the test birds and at the same intervals as the test birds. Two pens containing either 5 male or 5 female quail were used for each treatment and control group.

Observations: After dosing, the birds were observed multiple times on Day 0, and then twice daily for 14 days following dosing for mortality, toxicity symptoms and abnormal behavior. In addition, birds were observed for any signs of regurgitation. Body weights were measured individually the day prior to dosing (Day -1) and on Day 3, 7, and 14 of the test. Feed consumption was determined by pen for approximately 24-hour intervals from Day 0 to Day 1, Day 1 to Day 2 and Day 2 to Day 3. Average daily feed consumption was determined by pen for the dosage group and the control group for Days 3–7, and 7–14. At study termination, three birds from each group were subjected to a gross necropsy.

Statistical calculations: An LD_{50} value was not able to be calculated since no treatment group resulted in greater than 50% mortality. The bw and bw change by interval was compared between the TDS group and the two control groups by Student's t-test using Microsoft Excel®. The sample unit was the individual bird within each experimental group and the comparison was made by sex and by group.

Results

There were no mortalities in the control or treatment groups. When compared to the negative control group, there were no apparent treatment-related differences in mean bw, mean bw changes, or feed consumption for the males and females in 3500 mg/kg dosage level, except for significantly higher (Student's t-test, $p<0.01$) mean body weight change for the males in the TDS group than the males in the negative control group from Day -1 to Day 14 of the study. There were no remarkable findings for the birds subjected to gross necropsy from any group.

Validity criteria

There was no evidence of regurgitation by any bird in any of the treatment groups. All birds in the negative control group, CDS group and TDS group were normal in appearance and behavior for the duration of the study.

All validity criteria according to OCSPP 850.2100 were fulfilled, as no mortalities were observed in the control groups.

Results from the dose confirmation demonstrated that the Cry1Da_7 protein was homogeneous and stable under conditions of administration.

Conclusion

The acute oral LD_{50} value for northern bobwhite quail exposed to formulated Cry1B.868 protein as a single oral dose was determined to be greater than 3500 mg Cry1B.868 protein/kg, the highest dosage level tested. The no-mortality level was 3500 mg Cry1B.868 protein/kg bw.

J.15. Evaluation of the Potential effects of Cry1B.868 Protein on the Mouse, *Mus musculus*

Materials and Methods

Materials

Test material and test dose solution:

Test item:	Cry1B.868 protein
Description:	Light brown liquid
Purity corrected concentration:	86.5 mg/mL
Control:	Bovine serum albumin (BSA)
Vehicle control:	Permeate collected during concentration of the test substance was thoroughly dialized into 10 mM sodium carbonate/bicarbonate buffer. Dialized permeate was used as vehicle control for this study.

Test organisms:

Species:	CD-1 mice (*Mus musculus*)
Age:	8 weeks
Sex:	10 males/10 females per treatment group
Weight:	29.6-39.5 g males/ 22.9-31.2 g females
Diet/Food:	PMI Nutrition International Certified Rodent Chow No. 5002, *ad libitum*
Acclimation Period:	6 days

Environmental conditions:

Temperature:	21°C
Humidity:	48-57%
Photoperiod:	12 hours light / 12 hours dark

Study Design and Methods

Experimental treatments: After an acclimation period of 6 days in their designated housing, ten male and ten female mice were indiscriminately assigned to the treatment and control groups. Dosages used in this study were 0 mg of vehicle dose substance (VDS) per kg, 5000 mg of control (BSA) dose substance (CDS) per kg and 5000 mg of test (MON 95379) dose substance (TDS) per

kg. Any assigned animal considered unsuitable for use in the study was replaced by an alternate animal obtained from the same shipment and maintained under the same environmental conditions. The animals were housed individually throughout the study in polycarbonate cages containing appropriate bedding equipped with an automatic watering valve. Animals were provided with enrichment items and edible treats for psychological/environmental enrichment, except when interrupted by study procedures/activities. On Day 0, the animals chosen for use on study were weighed and fasted approximately 3 to 4 hours prior to dose administration. The test, control, and vehicle dosing solutions were administered in 2 doses (approximately 3 to 4 hours apart), each with a dose volume of 33.3 mL/kg body weight by oral gavage on Day 0 to the appropriate group of 10 males and 10 females. Individual doses were calculated based on the animal's non-fasted (Day 0) body weght. The animals were fasted between doses and were returned to feed following the second dose.

Observations: After dosing, the animals were observed for general health/mortality and moribundity twice daily, once in the morning and afternoon, throughout the study. The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, and gross necropsy findings. Each animal was observed for clinical signs once prior to study start, once prior to dosing on Day 0, a minimum of 2 times postdose on Day 0 (1 within 1 hour of the first dose and 1 approximately 2 hours following the second dose), and daily thereafter (Days 1 to 14). A final detailed clinical observation was performed for each animal on the day of scheduled euthanasia (Day 14). Body weights were measured individually once prior to study start, prior to fasting (Day 0), prior to dosing (Day 0, fasted), and on Days 7 and 14 of the test. Feed consumption was determined for each animal on Days 0, 7, and 14 and was reported for the intervals from Study Days 0 to 7 and 7 to 14. At study termination, three animals from each group were subjected to a gross necropsy.

Statistical calculations: Body weight, body weight change and food consumption were compared between the three treatment groups using an overall one-way ANOVA F-test or Kruskal-Wallis test (if parametric assumptions were not met) at the 5% significance level, but excluded semi-quantitative data, and any group with less than 3 observations. Levene's test was used to assess the homogeneity of group variances parametric assumption at the 5% significance level. Pairwise comparisons were conducted between TDS and the control groups using a two-sided Dunnett's or Dunn's test, respectively, if the overall test was significant. All significant pairwise comparisons were reported at the 0.1%, 1%, and 5% significance levels.

Results

There were no mortalities in the control or treatment groups throughout the study. There were no test substance-related clinical signs during the study. Clinical findings included piloerection in 3/10 TDS males. This finding was considered incidental as there were no other correlating observations. All other clinical findings noted were considered normal for animals of this age and

strain. There were no test substance-related effects on body weights, body weight gains, food consumption or gross necropsy findings noted during the study.

Observations

The analytical tests established stability and homogeneity of the test and control dosing solutions administered in the mouse oral acute gavage toxicity with Cry1B.868 protein.

Conclusion

There were no test substance-related adverse effects of Cry1B.868 protein when administered by oral gavage at a dose of 5000 mg/kg body weight in male and female CD-1 mice on mortality, clinical signs, body weights, body weight gains, food consumption or gross pathology findings. Therefore, the NOAEL for Cry1B.868 protein was 5000 mg/kg body weight.

J.16. Evaluation of the Potential Effects of Cry1Da_7 Protein on Larvae of the Ladybird Beetle, *Coleomegilla maculata*

Materials and Methods

Materials

Test material:	
Test item:	Cry1Da_7 protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	100 µg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment
Test organisms:	
Species:	*Coleomegilla maculata*
Age:	First instar, <24 hour old
Diet/Food:	Artificial diet provided *ad libitum*
Environmental conditions:	
Temperature:	27 ºC
Relative Humidity:	60%
Photoperiod:	14 hours light / 10 hours dark

Study Design and Methods

Experimental treatments: Cry1Da_7 protein (in a buffer vehicle) was incorporated into an artificial agar-based diet at concentrations of 50 and 415.1 µg Cry1Da_7 protein/g diet. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate buffer solution, pH 10.5 incorporated into the diet at the same level as in the 415.1 µg Cry1Da_7 protein diet), and a positive control diet containing potassium arsenate at 100 µg/g diet were included in the test. Each treatment was incorporated into a diet medium and provided *ad libitum* and continuously to individually housed *C. maculata* larvae (target n = 30 per treatment) until the test larvae develop into adults. Each test arena consisted of an inverted 60 mm × 15 mm Petri dish containing a filter paper, moistened with approximately two drops of deionized water, and a Post-it® flag (25.4 mm x 43.2 mm). Freshly-defrosted treatment diet (approximately one 1/8 section of a 1 mL diet aliquot) was placed on Post-it® flag in each test arena and renewed every 48 to 72 hours. A targeted two additional drops of water were added to each arena daily. The other test arena contents (e.g. frass) were removed or replaced as needed. There were approximately equal numbers of larvae per treatment per day.

Observations: Following the initiation of the diet exposures, daily observations were made to record larval survival and development, and body weight of the newly-emerged adults.

Statistical calculations: Fisher's exact test was performed to compare the survival between each treatment with the buffer control. Treatment effects on the time of development to emerged adult and adult body weight were analyzed with a linear model using SAS PROC Mixed. Pair-wise comparisons with the buffer control were defined within the linear model and tested using t-tests with significant difference at $\alpha = 0.05$.

Results

C. maculata larval survival was 93% in the negative control and 93% in the buffer control, compared with 86.2% and 100.0% survival in the 50 and 415.1 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in survival between either test treatment level and the buffer control ($p>0.05$). The positive control treatment resulted in 100% mortality.

The mean adult body weight was 10.5 mg in the negative control and 10.5 mg in the buffer control, compared with 11.1 and 10.1 mg in the 50 and 415.1 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in mean adult mass between either treatment level and the buffer control ($p>0.05$). No insects developed into adults in the positive control treatment.

The mean development time was 20.1 days in the negative control and 20.2 days in the buffer control, compared with 21.0 days in both the 50 µg Cry1Da_7/g and 415.1 µg Cry1Da_7/g protein

treatment levels. There was a significant increase in development time (<1 day) between both test treatment levels and the buffer control ($p<0.05$).

Assay Validity Criteria

The survival of *C. maculata* larvae in both the negative (water) and buffer control groups was 93.3%, indicating there was no adverse effect from the buffer on survival. These results met assay acceptance criteria of having ≥80% survival in the control. The positive control treatment resulted in 100% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis confirmed that the Cry1Da_7 protein in the 415.1 µg Cry1Da_7/g diet treatment level was homogeneous and was stable under test and frozen storage conditions. However, the targeted concentration could not be confirmed in the 415.1 µg Cry1Da_7/g diet. Because the Cry1Da_7 test substance incorporated into the *C. maculata* diet had lower than the expected level of biological activity in the 415.1 µg Cry1Da_7/g diet, the concentration was corrected based on the difference in activity between the reference and combined Day 0 test diet observed in the dose confirmation study. The effective concentration was calculated to be 150 µg Cry1Da_7/g diet. While diets from the 50 µg Cry1Da_7/g diet treatment level were not evaluated for dose, homogeneity, and stability, the same correction factor was applied to this treatment level resulting in a calculated effective concentration of 18 µg Cry1Da_7/g diet.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous dietary exposure to Cry1Da_7 protein at concentrations of either 18 or 150 µg/ml diet had no effects on the survival or adult mass of *C. maculata*. A statistically significant increase in development time (<1d) was observed for the Cry1Da_7 treatments at both concentrations. However, given the relatively small magnitude of the effect and the lack of effects in other study endpoints, the increase in development time was not considered to be adverse. The LC_{50} was >150 µg Cry1Da_7 protein/g diet and the NOAEC was 150 µg Cry1Da_7 protein/g diet, the highest concentration tested.

J.17. Evaluation of the Potential Effects of Cry1Da_7 Protein on Larvae of the Carabid Beetle, *Poecilus cupreus*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7 protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Reference and/or positive control:	Teflubenzuron Pestanal (Sigma-Aldrich)
Dietery concentration:	32 µg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the diet at the same rate as for the 400 µg/g test substance diet

Test organisms:

Species:	*Poecilus cupreus* larvae
Age:	1-2 d
Diet/Food:	Artificial diet provided *ad libitum*
Acclimation Period:	11 days after hatching

Environmental conditions:

Temperature:	19.5-21.6 ºC
Relative humidity:	71-80%
Photoperiod:	16 hours light / 8 hours dark
Illumination:	742 lux

Study Design and Methods

Experimental treatments: Cry1Da_7 protein (in a buffer vehicle) was incorporated into a base diet mixture at two separate treatment concentrations, equivalent to 50 and 400 µg Cry1Da_7 protein/g diet. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate, pH 10.5 incorporated into the diet at the same rate as for the 400 µg/g test substance diet), and a positive control of teflubenzuron at 32 µg/g diet to confirm the effectiveness of the dietary feeding, were included in the study. The test arenas comprised lidded clear plastic tubes, each containing one beetle larva placed onto the surface of 25 g (dry weight) of a standard sandy soil moistened to 25% ± 5% of its maximum water-holding capacity (WHC). For the study, the soil-filled plastic tubes were stored upright in plastic boxes (one box per treatment), with lids containing 8 holes covered with nylon netting for ventillation and floor lined

with capillary matting to raise the relative humidity around the tubes. Freshly-defrosted aliquots of appropriate treatment diets were provided to 24-48 hour old *P. cupreus* larvae (n = 40 per treatment) and diets were refreshed daily until all larvae had pupated or 21 days had passed.

Observations: The endpoints of the study were pre-imaginal mortality of the test insects, development time to adult emergence, and body weight of the emerged adult beetles. The condition of visible beetle larvae was assessed three times per week for the first three weeks of the study, and twice each week thereafter until all larvae had pupated. Fresh body weight of adult beetles was recorded after the elytra had hardened. The study ended 45 DAT when it was determined that no more adults would emerge.

Statistical calculations: The overall percentage pre-imaginal mortality in each treatment was determined and the data corrected for any control deaths using Abbott's formula (Abbott, 1925). Multiple sequentially-rejective Fisher test after Bonferroni-Holm correction (one-sided, > control, $\alpha = 0.05$) was used to compare mortalities in the test substance and the buffer control treatment. The positive control treatment was compared to the buffer control by Fisher's exact binomial test (one-sided, > control, $\alpha = 0.05$). Adult body weight was evaluated with a Dunnett's multiple t-test (one-sided, < control $\alpha = 0.05$) comparing the test treatments to the buffer control. Effects on development time were evaluated with a two-sample Mann Whitney *U*-test (one-sided, > control $\alpha = 0.05$) comparing the test treatments to the buffer control.

Results

Pre-imaginal mortality was 15% in the negative control and 10% in the buffer control, compared with 5% and 7.5% mortality in the 50 and 400 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$). No abnormal larvae, pupae or adults were seen, in any of the treatments. The positive control treatment resulted in 100% pre-imaginal mortality (100% corrected) and this treatment differed significantly from the buffer control.

The mean emergence time for beetles surviving was 39.1 days in the negative control and 38.4 days in the buffer control, compared with 39.3 days and 37.3 days in the 50 and 400 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in development time between either test treatment level and the buffer control ($p>0.05$).

The mean body weight of individual beetles taken soon after their emergence was 68.0 mg in the negative control and 69.4 mg in the buffer control, compared with 68.2 and 69.8 mg in the 50 and 400 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in mean body weight between either test treatment level and the buffer control ($p>0.05$).

Assay Validity Criteria

The mortality of *P. cupreus* was 15% in the negative control and 10% in the buffer control, indicating there was no adverse effect from the buffer on survival. These results met the assay acceptance criteria of having ≤20% mortality in the control. The positive control had 100% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis performed for the 400 µg Cry1Da_7 protien/g diet demonstrated the Cry1Da_7 test substance incorporated into diet had the expected level of biological activity, was considered homogeneous, and was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous dietary exposure to Cry1Da_7 protein at concentrations of either 50 µg or 400 µg Cry1Da_7/g diet had no effects on on the survival, development time or adult body weight of the ground-dwelling carabid beetle, *Poecilus cupreus*. The LC_{50} was >400 µg Cry1Da_7 protein/g diet and the NOAEC was 400 µg Cry1Da_7/g diet, the highest concentration tested.

J.18. Evaluation of the Potential effects of Cry1Da_7 Protein on Nymphs of the Big-Eyed Bug, *Geocoris punctipes*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7 protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	100 µg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment

Test organisms:

Species:	*Geocoris punctipes*
Age:	1 d
Diet/Food:	*Ephestia kuehniella* eggs and a slice of green bean pod provided *ad libitum*
Acclimation Period:	1 d

Environmental conditions:

Temperature:	25 ºC
Relative Humidity:	50%
Photoperiod:	14 hours light / 10 hours dark

Study Design and Methods

Experimental treatments: Cry1Da_7 protein (in a buffer vehicle) was incorporated into a base diet mixture at two separate treatment concentrations, equivalent to 50 and 500 µg Cry1Da_7 protein/g diet each encapsulated to form a dome stored at -20°C. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate, pH 10.5 incorporated into the diet at the same rate as for the 500 µg/g test substance diet), and a positive control diet containing potassium arsenate at 100 µg/g diet were included in the study. The nymphs after one-day acclimation were individually transferred into test arenas (inverted Falcon petri dishes, 60×15mm, Corning, NY) and impartially assigned to each of the treatments by providing appropriate treatment diets. Two freshly-defrosted encapsulated diets (domes) of the appropriate

treatment diet were provided in each test arena and renewed every 48 hours. The nymphs were allowed to feed *ad libitum* throughout the study duration. Each diet treatment consisted of a total of 30 nymphs individually housed in inverted petri dishes lined with a piece of filter paper on the bottom. One drop of distilled water was added on the filter paper every day at the time of observation to moisten the filter paper.

Observations: Daily observations were made to record nymph survival, the time taken for the nymphs to develop into adulthood, and the fresh body weight of the newly-emerged adults.

Statistical calculations: Fisher's Exact test was performed to compare the survival between predefined treatments. Treatment effects on the time of development to adult and adult body mass were analyzed with a linear model using SAS PROC Mixed. Pair-wise comparisons with the buffer control were defined within the linear model and tested using t-tests with significant difference at $\alpha = 0.05$.

Results

G. punctipes nymph survival was 86.7% in the negative control and 96.7% in the buffer control diet treatments, compared with 89.7% and 93.3% survival in the 50 and 500 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$). The positive control treatment resulted in 100% mortality.

All surviving nymphs completed the development into adulthood. The mean nymph development time to adults was 31.0 days in the negative control and 30.3 days in the buffer control, compared with 30.3 days and 30.5 days in the 50 and 500 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in development time between either test treatment level and the buffer control ($p>0.05$).

The mean body weight of newly-emerged adults was 4.4 mg in the negative control, 4.3 mg in the buffer control, compared with 4.5 mg in both test treatment levels. There was no significant differences in mean body weight between either test treatment level and the buffer control ($p>0.05$).

Assay Validity Criteria

The survival of *G. punctipes* nymphs was 86.7% in the negative control and 96.7% in the buffer control, indicating there was no adverse effect from the buffer on survival. These results met the assay acceptance criteria of having ≥80% survival in the control. The positive control resulted in 100% mortality demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated that the Cry1Da_7 protein in the treatment diet had the expected level of biological activity and was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous dietary exposure to Cry1Da_7 protein at concentrations of either 50 or 500 µg Cry1Da_7 protein/g diet had no effects on either the survival or development of the *G. punctipes* nymphs. The LC_{50} was >500 µg Cry1Da_7 protein/g diet and the NOAEC was 500 µg Cry1Da_7 protein/g diet, the highest concentration tested.

J.19. Evaluation of the Potential Effects of Cry1Da_7 Protein on Adults of the Parasitoid Wasp, *Pediobius foveolatus*

Materials and Methods

Materials

Test material:	
Test item:	Cry1Da_7 protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	200 µg/ml diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment
Test organisms:	
Species:	*Pediobius foveolatus*
Age:	1 d
Diet/Food:	30% honey/water (v/v) solution
Acclimation Period:	24 h
Environmental conditions:	
Temperature:	25 ºC
Relative Humidity:	70%
Photoperiod:	14 hours light / 10 hours dark

Study Design and Methods

Experimental treatments: Cry1Da_7 protein (in a buffer vehicle) was incorporated into 30% honey/purified water solution at two nominal concentrations of 50 and 500 µg/ml 30% honey/purified water solution, respectively. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate, pH 10.5 incorporated into the diet at the same rate as for the 500 µg/ml test substance diet), and a positive control diet containing potassium arsenate at 200 µg/ml diet were included in the study. The newly emerged *P. foveolatus* adults produced in laboratory from the parasitized *E. varivestis* larvae were provided a 30% honey/water (v/v) solution and were impartially transferred into 162 cm^2 cell culture flasks. After approximately 24 hours of acclimation, 15 adults in each flask were impartially assigned to the five treatments and provided with appropriate treatment diet in two screened feeding dishes, replaced every two days. Each treatment included four replicates. The wasp adults were allowed to feed *ad libitum* throughout the 21 days feeding period.

Observations: All wasps in all treatments were observed for mortality/survival every two days at the time of diet replacement. Dead wasps, if any, were removed from the test arenas and recorded.

Statistical calculations: Statistical analyses were not performed since no mortality was recorded in either Cry1Da_7 treatment level as well as the negative and buffer controls and 100% mortality was recorded in positive control treatment.

Results

P. foveolatus adult survival was 100% in both 50 and 500 µg Cry1Da_7 protein/ml diet treatment levels as well as the negative and buffer controls. The positive control treatment resulted in 100% mortality (0% survival).

Assay Validity Criteria

The survival of *P. foveolatus* larvae in both the negative control and buffer control was 100%, indicating there was no adverse effect from the buffer on survival. These results met assay acceptance criteria of having ≥80% survival in the control. The positive control treatment resulted in 100% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated that the Cry1Da_7 protein in the treatment diet had the expected level of biological activity, and was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous dietary exposure to Cry1Da_7 protein at concentrations of either 50 or 500 µg Cry1Da_7 protein/ml diet for 21 days

had no effects on the survival of the *P. foveolatus* adults. The LC_{50} was >500 µg Cry1Da_7 protein/ml diet, the highest concentration tested.

J.20. Evaluation of the Potential Effects of Cry1Da_7 Protein on Adults of the Green Lacewing, *Chrysoperla carnea*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7 protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Reference and/or positive control:	Dimethoate
Dietery concentration:	0.04 µg a.s./g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the diet at the same rate as for the 500 µg/g test substance diet

Test organisms:

Species:	*Chrysoperla carnea*
Age:	24 h
Sex:	10 males/10 females per treatment group
Diet/Food:	None

Environmental conditions:

Temperature:	23.8-25.4 ºC
Relative humidity:	70-78%
Photoperiod:	16 hours light / 8 hours dark
Illumination:	2000-4800 lux

Study Design and Methods

Experimental treatments: Cry1Da_7 protein (in a buffer vehicle) was incorporated into an artificial agar-based diet at concentrations of 50 and 500 µg Cry1Da_7 protein/g diet. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate buffer solution, pH 10.5 incorporated into the diet at the same level as in the 500 µg Cry1Da_7 protein diet), and a positive control diet containing dimethoate at 0.04 µg/g diet to confirm the effectiveness of the dietary feeding, were included in the test. The test arenas comprised clear polystyrene boxes with close-fitting lids. The dishes of the treated diet, and dishes of honey-water and water-only, were

placed on the floor of each box. For each treatment there were two replicate boxes, each containing 20 *Chrysoperla carnea* adult lacewings (i.e. 40 per treatment) with 10 female and 10 male lacewings impartially assigned to each test arena. Any insects that appeared to have been damaged during the transfer process were replaced immediately. Freshly-defrosted aliquots of appropriate treatment diets were provided to newly-emerged adult lacewings three times per week, for two weeks.

Observations: The endpoint of the study was an assessment of the adult mortality. The adults were assessed every 1-3 days for 14 days and finally at 14 days after treatment, from which the overall percentage mortality in each treatment was calculated.

Statistical calculations: The percentage adult mortality in each treatment was determined and the data corrected for any insect deaths in the buffer control using Abbott's formula (Abbott, 1925). Mortality was evaluated with a multiple sequentially-rejective Fisher test after Bonferroni-Holm correction (one-sided, > control, $\alpha = 0.05$) comparing mortalities in the test substance and the buffer control. The positive control treatment was compared to the buffer control by Fisher's exact binomial test (one-sided, > control, $\alpha = 0.05$).

Results

Mortality of *C. carnea* adults was 17.5% in the negative control and 10% mortality in the buffer control, compared with 30% and 7.5% mortality in the 50 µg and 500 µg Cry1Da_7 protein/g diet treatment levels, respectively. When corrected against the buffer control, mortality was 22.2% and -2.8% in the 50 µg and 500 µg Cry1Da_7 protein/g diet treatment levels, respectively. There was no significant difference in mortality between the 500 µg Cry1Da_7/g diet treatment level and the buffer control ($p>0.05$). The 50 µg Cry1Da_7 protein/g diet treatment showed significantly increased mortality compared to the buffer control ($p = 0.024$). However, since no adverse effects were observed in the 500 µg Cry1Da_7 protein/g diet treatment and the concentration in diet was confirmed by sensitive insect assay, it was considered that the statistically significant increase in mortality at the 50 µg Cry1Da_7 protein/g diet treatment level was not due to a treatment effect. The positive control treatment resulted in 77.5% mortality and this treatment differed significantly from the buffer control ($p<0.05$).

Assay Validity Criteria

Mortality of *C. carnea* was 17.5% in the negative control and 10.0% in the buffer control, indicating there was no adverse effect of the buffer on survival. These results met assay acceptance criteria of having ≤20% mortality in the control. The positive control had 77.5% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis by sensitive insect bioassay demonstrated that the Cry1Da_7 protein at the 500 µg Cry1Da_7/g diet treatment level was biologically active, homogenous throughout the test diet and was stable under test and frozen storage conditions. For the 50 µg Cry1Da_7/g

diet treatment level, the Cry1Da_7 protein was confirmed to be homogenous throughout the test diet and was stable under test conditions, but did not have the expected level of biological activity. Therefore, 50 µg Cry1Da_7/g diet treatment level was corrected to an effective concentration of 27 µg Cry1Da_7 protein/g of diet.

Conclusion

Under laboratory test conditions, continuous dietary exposure of Cry1Da_7 protein to adults of the green lacewing, *C. carnea* over 14 days resulted in no treatment-related adverse effects on survival at either 27 (dose-corrected) or 500 µg protein/g diet. The LC_{50} was >500 µg Cry1Da_7 protein/ml diet, the highest concentration tested.

J.21. Evaluation of the Potential Effects of Cry1Da_7 Protein on Larvae of the Green Lacewing, *Chrysoperla rufilabris*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7 protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Reference and/or positive control:	Potassium arsenate
Dietery concentration:	0.243 mg/g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the diet at the same rate as for the 500 mg/kg test substance diet

Test organisms:

Species:	*Chrysoperla rufilabris*
Age:	Second instar larvae
Diet/Food:	Untreated diet and water soaked cotton balls
Acclimation Period:	24 hours

Environmental conditions:

Temperature:	24-26ºC
Relative humidity:	55-65%
Photoperiod:	16 hours light / 8 hours dark
Illumination:	420-790 lux

Study Design and Methods

Experimental treatments: Second instar *Chrysoperla rufilabris* larvae (3-5 days from egg hatch) were exposed to six treatments, Cry1Da_7 protein at 500 µg protein/g diet, Cry1Da_7 protein at 100 µg protein/g diet, Cry1Da_7 protein at 20 µg protein/g diet, a negative control consisting of meat-based diet without any test substance or buffer added, buffer control consisting of 25 mM sodium carbonate buffer solution (pH 11.5) incorporated into the diet at the same rate as for the 500 µg/g test substance diet, and a positive control of potassium arsenate at 0.243 µg product/g diet to confirm the effectiveness of the dietary feeding for 24 days. The test vessels comprised Petri dishes with lids. Lacewing larvae were acclimated for 24 hours prior to study initiation and were impartially allocated to the test vessels. Each treatment and control had 40 replicate test vessels with each vessel containing one lacewing larva. Individual vessels were randomly assigned to the treatments using computer generated random numbers. Test vessels were then randomly placed within the environmental chamber during the exposure. At study initiation, one freshly-defrosted 50 µL pellet of the appropriate control or treatment level was added to each test vessel along with one moistened small cotton ball with both being replaced at every 48-hour interval until organisms reached pupal state, when organisms do not actively feed on diet. The study was terminated on day 24 and pupae that had not emerged were considered dead.

Observations: The endpoints of the study were percentage pre-imaginal survival, development time from larvae to emerged adult, and adult weight at emergence. The health of the organisms was observed and recorded daily. Pupation and adult emergence were recorded when observed.

Statistical calculations: The treatment data were tested for normality and homogeneity of variance using the appropriate qualifying test. Fisher's exact test with Bonferroni-Holm correction was used to compare survival between the treatment and negative control groups. Effects on development time were evaluated with a Dunnett's multiple comparison test comparing test treatments to the negative control. Adult bw was evaluated with a Jonckheere-Terpstra's Step-down test comparing the test treatments and the negative control. All comparisons were made at ≥95% level of certainty ($\alpha = 0.05$) and compared on a per replicate basis.

Results

The mean pre-imaginal survival was 85% in the negative control and 88% in the buffer control, compared with 75, 75, and 73% in the 20, 100, and 500 mg/kg treatment levels, respectively. There were no significant reductions in pre-imaginal survival among organisms exposed to the test treatment levels compared to the negative control ($p>0.05$).

The mean weight for adults at emergence was 0.0076 in the negative control and 0.0077 in the buffer control, compared with 0.0072, 0.0071, and 0.0076 g in the 20, 100, and 500 mg/kg treatment levels, respectively. There were no significant reductions in emergence weight among organisms exposed to the test treatment levels compared to the negative control ($p>0.05$).

The mean development time from larvae to emerged adult was 19 for the negative control, buffer control, 20, 100, and 500 mg/kg treatment levels. There were no significant reductions in time from larvae to emerged adult among organisms exposed to the test treatment levels compared to the negative control ($p>0.05$).

Assay Validity Criteria

The survival of *C. rufilabris* was 85% in the negative control and 88% in the buffer control group, indicating there was no adverse effect from the buffer on survival. These results met acceptance criteria of having ≥80% survival in the control. The positive control had 100% larval mortality demonstrating sensitivity of the test species.

The results of the diet analysis demonstrate that the Cry1Da_7 test substance incorporated into the test substance treatment diets had the expected level of biological activity, was homogeneous throughout the test substance diet, was stable under test and frozen storage conditions.

Conclusion

Under laboratory test conditions, Cry1Da_7 protein had no statistically significant effects on the survival and development of larvae or adult weight of the green lacewing, *Chrysoperla rufilabris* at concentrations of 20 µg, 100 µg, and 500 µg Cry1Da_7/g diet fed continuously during their development for 24 days. The LC_{50} was >500 µg Cry1Da_7 protein/g diet and the NOAEC was 500 µg Cry1Da_7/g diet, the highest concentration tested.

J.22. Evaluation of the Potential Effects of Cry1Da_7 Protein on the Springtail, *Folsomia candida*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7 protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Reference and/or positive control:	Teflubenzuron
Dietery concentration:	0.1 mg per g diet
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment

Test organisms:

Species:	*Folsomia candida*
Age:	12 d
Diet/Food:	Artificial diet provided *ad libitum*
Acclimation Period:	11 days after hatching

Environmental conditions:

Temperature:	19.3-21.0 ºC
Photoperiod:	16 hours light / 8 hours dark
Illumination:	560-710 lux

Study Design and Methods

Experimental treatments: Cry1Da_7 protein (in a buffer vehicle) was incorporated into a diet medium of inactivated yeast at two separate treatment concentrations, equivalent to 50 and 500 µg Cry1Da_7 protein/g diet. Additionally, a buffer control diet to which 25 mM sodium carbonate buffer solution (pH 10.5) was added at an equivalent concentration as in the 500 µg Cry1Da_7 protein/g treatment, a negative control diet to which only purified water was added, and a positive control diet treated with a technical-grade sample of teflubenzuron, at a concentration equivalent to 0.1 mg/g diet were included in the study. Freshly-defrosted aliquots of appropriate treatment diets were provided continuously to batches of confined springtails over a 28-day period. The test arenas comprised lidded jars, the bases of which were lined with a plaster-of-Paris and charcoal substrate. Ten juvenile springtails (12 days old) were placed in each jar (n = 4 per treatment) and

were fed *ad libitum* throughout the study. Freshly-treated aliquots of appropriate treatment diet were provided on the day of study initiation (Day 0) and additional freshly-defrosted aliquots were then provided every 2-3 days. At Day 28, the numbers of surviving springtails and their offspring (progeny) were recorded for each replicate arena.

Observations: For the study, the numbers both of surviving adults and F1 progeny in each test arena were recorded at 28 days after treatment (DAT). It was assumed that any adult springtails that were recovered would have been those originally introduced and that any shortfall in the original number was an indication that they had died during the study. The numbers of surviving springtails were used to calculate the percentage mortality of the springtails originally introduced in each treatment.

Statistical calculations: The overall percentage mortality in each treatment was determined and the data corrected for any control deaths using Abbott's formula (Abbott 1925). Multiple sequentially-rejective Fisher test after Bonferroni-Holm correction (one-sided greater, $\alpha = 0.05$) was used to compare mortalities in the individual test substance treatments with the buffer control. Following a check for normal distribution of the data (Shapiro-Wilk test, $\alpha = 0.05$) and for equality of variances (Levene's test, $\alpha = 0.05$), test substance treatments were compared to the buffer control by multiple sequentially-rejective t-test after Bonferroni-Holm correction (one-sided smaller, $\alpha = 0.05$). The positive control treatment was compared to the buffer control by Student t-test for homogeneous variances (one-sided smaller, $\alpha = 0.05$).

Results

After 28 days, mortality was 0% in both the negative and buffer controls, compared with 3% and 0% mortality in the 50 and 500 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. When adjusted for the buffer control treatment, the test substance treatments resulted in 3% and 0% corrected mortality in the 500 and 50 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$). The positive control treatment resulted in 100% mortality (100% corrected) and this treatment differed significantly from the buffer control ($p>0.05$).

The mean number of progeny produced per treatment was 557 in the negative control and 560 in the buffer control, compared with 531 and 547 in the 50 and 500 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in reproduction between either Cry1Da_7 treatment level and the buffer control ($p>0.05$).

Assay Validity Criteria

The mortality of *F. candida* was 0% in both the negative and buffer controls, indicating there was no adverse effect from the buffer on survival. These results met the assay acceptance criteria of having ≤20% mortality in the control. The mean number of juveniles recorded in the negative

control treatment was at least 100 per replicate at the end of the study and the coefficient of variation of reproduction in the negative control was ≤30% meeting acceptance criteria. The positive control treatment resulted in 100% mortality, demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated that the test substance, Cry1Da_7 protein in test diet had the expected level of biological activity, was homogenous throughout the freshly-prepared test diet and was stabile under test and storage conditions.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous exposure to Cry1Da_7 protein for 28 days at concentrations of either 50 or 500 µg Cry1Da_7 protein/g diet had no effects on either the survival or reproduction rate of the springtail *Folsomia candida*. The LC_{50} was >500 µg Cry1Da_7 protein/g diet and the NOAEC was 500 µg Cry1Da_7 protein/g diet, the highest concentration tested.

J.23. Evaluation of the Potential effects of Cry1Da_7 Protein on the Earthworm, *Eisenia andrei*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7protein
Description:	Clear colorless solution
Purity corrected concentration:	3.3 mg/mL
Vehicle and/or positive control:	2-chloroacetamide (tested in a separate study)
Buffer control:	25 mM sodium carbonate buffer solution, (pH 10.5) incorporated into the soil to achieve the same concentration of buffer per g soil as used for the test substance treatments

Test organisms:

Species:	*Eisenia andrei*
Age:	~7.5 months
Diet/Food:	None
Acclimation Period:	1 day

Environmental conditions:

Temperature:	20.2-20.9 ºC
Photoperiod:	24 hours light
Illumination:	520-600 lux

Study Design and Methods

Experimental treatments: The Cry1Da_7 protein held in a buffer solution was incorporated into an artificial soil medium at two separate treatment rates, equivalent to 50 and 500 µg Cry1Da_7protein/g soil dry weight. For the study, these two treatments were compared to a control soil treated with either 25 mM sodium carbonate buffer solution, pH 10.5 at the same concentration as used for the test substance treatments (buffer control) or with purified water (negative control). To confirm sensitivity of the test species, a positive control treatment of 2-chloroacetamide was evaluated in a separate, GLP-compliant study within 2 months prior to the start of the study. The artificial soil substrate (containing 10% w/w peat), was moistened to 50% of its pre-determined maximum water-holding capacity at the time of treatment. The treated soil was held within 1-L-capacity lidded jars (n = 4 per treatment) and 10 adult *E. andrei* (approx. 7.5 months old, with an individual fresh weight of 358-598 mg and with a visible clitellum) were weighed and introduced

into each jar immediately after treatment application. Each treatment was replicated four times for a total of 40 earthworms exposed per treatment. No food was provided throughout the duration of the study.

Observations: For the study, the numbers of surviving adult earthworms in each test arena were recorded at 7 and 14 DAT. It was assumed that any adult earthworms that were recovered would have been those originally introduced and that any shortfall in the original number was an indication that they had died during the study. The mean percentage change in weight of the earthworms in the controls and test-substance treatments over 14 days was calculated. The earthworms were gently cleaned using purified water and individually re-weighed at 14 DAT after removing excess moisture to determine the mean weight of individuals in each replicate. The percentage change in mean weight per replicate was derived by comparing the mean weight recorded at 0 DAT with that measured again at 14 DAT for earthworms in each replicate. The earthworms were also examined for any other harmful effects (e.g. behavioral abnormalities or open wounds) at 0, 7 and 14 DAT.

Statistical calculations: Since there was no mortality in the controls, it was unnecessary to correct for mean control mortality using Abbott's formula. Fisher's Exact binomial test with Bonferroni correction (one-sided, > control, $\alpha = 0.05$) was used to compare mortalities in the individual test treatments with the buffer control treatment. Following a check for normal distribution of the data (Shapiro-Wilk test, $\alpha = 0.05$) and for equality of variances (Levene's test, $\alpha = 0.05$), test treatments were compared to the buffer control by multiple sequentially-rejective t-test after Bonferroni-Holm correction (one-sided smaller, $\alpha = 0.05$).

Results

After 14 days, mortality was 3% in the negative control and 0% in the buffer control treatments, compared with no mortality in both the 50 and 500 µg Cry1Da_7protein/g soil dry weight treatment concentrations. Therer were no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$).

The mean percentage change in earthworm weight per treatment was equivalent to a decrease of 5.4% in the buffer control and 5.6% in the negative control, compared with an increase of 4.9% and a decrease of 1.4% in the 50 and 500 µg Cry1Da_7protein/g soil dry weight treatment concentrations, respectively. There was no significant decrease in body weight between the test treatments and the buffer control ($p>0.05$). At both 7 and 14 DAT, all of the earthworms in all of the treatments appeared healthy and active.

Assay Validity Criteria

The mortality of *Eisenia andrea* was 3% in the negative control and 0% in the buffer control group, indicating there was no adverse effect of the buffer on survival. These results met the assay acceptance criteria of ≤10% mortality in the control. The mean post-treatment weight of the negative control group decreased by <20% compared to the start of the study, meeting acceptance criteria.

As this was an acute study with a single application of the Cry1Da_7 protein, no direct measurement was made of test substance homogeneity in the soil. However, care was taken to mix the treated soil thoroughly. No measurement was made of the stability of the test substance in the soil, but the test earthworms were placed onto the soil immediately after the treatment of each arena.

Conclusion

Under laboratory test conditions, it was demonstrated that continuous exposure to Cry1Da_7 protein for 14 days in an artificial soil containing 10% w/w peat at concentrations of either 50 or 500 µg Cry1Da_7 protein/g soil dry weight had no significant adverse treatment effects on the survival or biomass of the earthworm *Eisenia andrei*. Additionally, no abnormal behaviors were observed in any of the treatments. The LC_{50} was >500 µg Cry1Da_7 protein/g soil dry weight and the NOAEC was 500 µg Cry1Da_7 protein/g soil dry weight, the highest concentration tested.

J.24. Evaluation of the Potential Effects of Cry1Da_7 Protein on Adults of the Honey Bee, *Apis mellifera*

Materials

Test material:

Test item:	Cry1Da_7
Description:	Liquid
Purity corrected concentration:	3.3 mg/mL
Positive control:	Dimethoate
Dietery concentration:	0.65 mg/kg diet
Buffer control:	25 mM sodium carbonate buffer solution (pH 10.5) incorporated into the diet to achieve the same concentration of buffer per g diet as used for the highest test substance treatment

Test organisms:

Species:	*Apis mellifera* L.
Age:	≤2d at dosing
Diet/Food:	50% sucrose/purified water solution provided *ad libitum*
Acclimation Period:	1 d

Environmental conditions:

Temperature:	33°C
Relative Humidity:	50-61 %
Photoperiod:	Maintained in the dark thoughout duration of study

Study Design and Methods

Experimental treatments: Honey bee adults, ≤2 days old, were exposed to five treatments, Cry1Da_7 protein at 10 µg/g in combination with 50% sucrose/purified water solution, Cry1Da_7 protein at 500 µg/g in combination with 50% sucrose/purified water solution. In addition, a negative control consisting of 50% sucrose/purified water solution only, buffer control consisting of test substance buffer (25 mM sodium carbonate buffer solution, pH 10.5) at a volume equivalent to the highest test substance concentration, and a positive control of dimethoate at 0.65 mg/kg to confirm the effectiveness of the dietary feeding, were included in the study. Five cages (replicates) for each five treatments comprised a total of 25 cages. Individual cages contained 10 adult bees obtained from a local hive mainatined by Eurofins that was apparently healthy, queen-right, and had not been treated for control of Varroa or pathogens within four weeks of the test. Once the honey bee adults were caged impartially, each cage was assigned to a treatment/replicate in a non-systematic fashion. After all adult bees were acclimated under red light with 50% (w/v) sucrose

solution provided *ad libitum*, each cage was provided with the appropriate treatment diet solution through two feeders (syringes, each containing ~2.5 mL diet) inserted through the lid of the test chamber and bees were allowed to feed *ad libitum*. Feeders were replaced daily and were weighed prior to placing them into the cages and again after removal 24 ± 2 hours later to measure the amount of diet consumed in each replicate. The study duration was 10 days with adult bees being observed daily for mortality, behavioral and toxicological responses.

Observations: All treatments were observed for mortality, behavioral and toxicological responses once within the first four hours after initial treatment and at approximately 24-hour intervals (from dosing) thereafter. Abnormal behavior was determined by comparing honey bees in the treatment groups with those in the negative control group. Dead honey bee adults remained in their respective cages until study completion to avoid unnecessary disturbances. Consumption per bee in each replicate was calculated for each day using the consumption data adjusted for evaporative loss and the total number of bees alive in each replicate at the start of each feeding period (day). The consumption per bee values for all the replicates within a treatment level were then averaged for each day. Replicates were no longer included in the average consumption calculations after reaching 100% mortality.

Statistical calculations: Dunnett's test was used to compare mortalities in the test treatments with the negative control.

Results

Mortality was 8% in the negative control and 4% in the buffer control, compared to 20% and 12% mortality in the 10 and 500 µg Cry1Da_7 protein/g diet treatment concentrations, respectively. There were no significant differences in mortality between test treatment levels and the negative control ($p>0.05$). All surviving bees appeared normal in the end of the study. Positive control group mean mortality was 100% at the end of the study.

Average daily consumption over the 10-day exposure period were 28, 29, 26, 30, and 11 mg diet/bee/day in the negative control, buffer control, 10 µg Cry1Da_7 protein/g, 500 µg Cry1Da_7 protein /g, and positive control groups, respectively. These results demonstrate that diet consumption was comparable in all groups, except positive control. The daily doses were calculated based on the average daily consumption values and were 0.26 and 15 µg Cry1Da_7 protein/bee/day during the test for the 10 µg Cry1Da_7 protein/g and 500 µg Cry1Da_7 protein/g treatment levels, respectively.

Assay Validity Criteria

The mortality of *Apis mellifera* adults was 8% in the negative control and 4% in the buffer control group, indicating there was no adverse effect from the buffer on survival. These results met assay acceptance criteria of ≤20% mortality in the control. The positive control had 100% mortality demonstrating sensitivity of the test species.

The results of the diet analysis demonstrated the Cry1Da_7 protein incorporated into the test treatment diets had the expected level of biological activity, was stable for 1 day under test conditions, was stable at 4 ℃ for 3 days, and was stable under frozen storage conditions.

Conclusion

Following oral exposure of adult honey bees to Cry1Da_7 protein for 10 days under laboratory test conditions, the LC_{50} was >500 µg Cry1Da_7 protein/g diet and the NOAEC value was 500 µg Cry1Da_7 protein/g, highest concentration tested.

J.25. Evaluation of the Potential Effects of Cry1Da_7 Protein on Larvae of the Honey Bee, *Apis mellifera*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7
Description:	Liquid
Purity corrected concentration:	3.3 mg/mL
Positive control:	Dimethoate
Dietery concentration:	7.42 µg/bee

Test organisms:

Species:	*Apis mellifera* L.
Age:	~6 d at dosing
Diet/Food:	Artificial diet (OECD 239)
Acclimation Period:	2 d after grafting

Environmental conditions:

Average Temperature:	34.47°C (Larval exposure phase)
	34.27°C (Pupal transfer to emergence)
Average Relative Humidity:	92.88 % (Larval exposure phase)
	76.80 % (Pupal transfer to emergence)
Photoperiod:	Maintained in the dark thoughout duration of study

Study Design and Methods

Experimental treatments: Cry1Da_7 protein (in a buffer vehicle) was incorporated into an artificial diet at concentrations of 10 and 500 µg Cry1Da_7 protein/g diet. Additionally, a negative control (purified water only), a buffer control (25 mM sodium carbonate buffer solution, pH 10.5 incorporated into the diet at the same level as in the 500 µg Cry1Da_7 protein diet), and a positive control diet containing dimethoate at 52.8 µg/ml diet to confirm the effectiveness of the dietary feeding, were included in the test. First instar larvae from three hives were transferred (grafted) to well plates containing untreated artificial diet and held in an incubator. Starting at two days after grafting, artificial diets containing the test or control substance were provided to larvae. Test diets were provided for a total of four days. Larvae were then held for up to 14 days after the completion of dosing in order to allow emergence of adult bees. Each treatment and control group contained 16 larvae from each of the three hives, for a total of 48 larvae per treatment group. Nominal test levels were 10 and 500 µg Cry1Da_7/g diet.

Observations: Starting on the first day of dosing (Day 3), larvae were observed daily until either mortality or adult emergence occurred. Cell cups containing dead larvae were removed from well plates after mortality was recorded. Observations of sublethal effects, including the presence of uneaten diet and larvae with reduced body size, were recorded on Days 7 and 8. Emerged bees from each replicate were checked to evaluate sublethal effects on morphology of emerged bees. By Day 20, approximately 14 days after final dosing, all bees had either emerged or died.

Statistical calculations: Dunnett's multiple comparison test was used to compare mortalities in the individual test treatments to the negative control. Mean larval survival and pupal survival were calculated but not be analyzed statistically. Sublethal effects were reported but not statistically analyzed.

Results

Mean mortality was 10% in the negative control and 17% in the buffer control and no significant difference was detected according to t-test ($p>0.05$). Mean mortality in the 10 and 500 µg a.i./g treatment groups were 15 and 13%, respectively. There was no significant differences in mortality between either test treatment level and the buffer control ($p>0.05$). In the end of the study, all emerged adult bees appeared healthy with no morphological deformities observed, except one bee with deformed wings in the buffer control.

Assay Validity Criteria

Cumulative larval mortality was less than 15% across replicates, adult emergence was higher than 70% on Day 20 in the negative control group, and larval mortality was higher than 50% by day 8 in the positive control group, meeting acceptance criteria.

The results of the diet analysis demonstrated the Cry1Da_7 protein incorporated into the test treatment diets had the expected level of biological activity, was homogeneous throughout the diet, was stable under test and frozen storage conditions.

Conclusion

Following dietary exposure of larval honey bees to Cry1Da_7 protein under laboratory test conditions, the LC50 was >500 µg Cry1Da_7 protein/g diet and the NOAEC value was 500 µg Cry1Da_7 protein/g, the highest concentration tested.

J.26. Evaluation of the Potential Effects of Cry1Da_7 Protein on the Northern Bobwhite Quail, *Colinus virginianus*

Materials and Methods

Materials

Test material:

Test item:	Cry1Da_7 lyophilized powder
Description:	Solid
Purity corrected concentration:	0.72 mg/mg lyophilized powder
Control:	Bovine serum albumin (BSA) lyophilized powder

Test organisms:

Species:	Northern bobwhite (*Colinus virginianus*)
Age:	27 weeks
Sex:	5 males/5 females per treatment group
Weight:	188-232 g
Diet/Food:	Cargill game bird ration, *ad libitum*
Acclimation Period:	8 weeks

Environmental conditions:

Temperature:	23.7°C (Range: 22.1-25.6°C)
Humidity:	59% (Range: 37-72%)
Photoperiod:	8 hours light / 16 hours dark
Illumination:	369 lux

Study Design and Methods

Experimental treatments: After an acclimation period of 8 weeks, ten adult northern bobwhite were indiscriminately assigned to the treatment and control groups. The test dose substance (TDS) and control dose substance (CDS) was dosed using capsules at a rate of 1000 mg product/kg of body weight. Nominal dosages used in this study were 0 mg/kg, 1000 mg of CDS/kg and 1000 mg TDS/kg. The birds were fasted for approximately 19 hours prior to dosing and individually weighed and dosed on the basis of mg of product/kg of body weight. At the experimental start, a capsule containing either the CDS or TDS was orally inserted into the crop of each bird. The negative control birds received an empty capsule of the same size used for the test birds. Two pens containing either 5 male or 5 female quail were used for each treatment and control group.

Observations: After dosing, the birds were observed multiple times on Day 0, and then twice daily for 14 days following dosing for mortality, toxicity symptoms and abnormal behavior. In addition, birds were observed for any signs of regurgitation. Body weights were measured individually the day prior to dosing (Day -1) and on Day 3, 7, and 14 of the study. Feed consumption was determined by pen for approximately 24-hour intervals from Day 0 to Day 1, Day 1 to Day 2 and Day 2 to Day 3. Average daily feed consumption was determined by pen for the dosage group and the control group for Days 3–7, and 7–14. At study termination, three birds from each group were subjected to a gross necropsy.

Statistical calculations: An LD_{50} value was not able to be calculated since no treatment group resulted in greater than 50% mortality. The body weight and body weight change by interval was compared between the TDS group and the two control groups by Student's t-test using Microsoft® Excel®. The sample unit was the individual bird within each experimental group and the comparison was made by sex and by group.

Results

There were no mortalities in the control or treatment groups. When compared to the negative control group and the CDS group, there were no apparent treatment-related differences in mean body weight, mean body weight changes, or feed consumption for the males and females in TDS group at 1000 mg/kg dosage level. The mean body weight change was significantly higher (Student's t-test, $p<0.01$) and lower (Student's t-test, $p<0.01$) for the females in the TDS group compared to the females in the negative control group from Day -1 to Day 3 and Day 3 to Day 7 of the test, respectively. There were no remarkable findings for the birds subjected to gross necropsy from any group, except one male bird of the three birds necropsied from the TDS group with feather loss and small lesions on the rump.

Assay Validity Criteria

There was no evidence of regurgitation by any bird in any of the treatment groups. All birds in the negative control group, CDS group and TDS group were normal in appearance and behavior for the duration of the study, except one male in the TDS group that was noted as being aggressive to pen mates on Day 3 of the study.

All validity criteria according to OCSPP 850.2100 were fulfilled, as no mortalities were observed in the control groups.

Results from the dose confirmation demonstrated that the Cry1Da_7 protein was homogeneous and stable under conditions of administration.

Conclusion

The acute oral LD_{50} value for northern bobwhite exposed to Cry1Da_7 protein as a single oral dose was determined to be greater than 1000 mg Cry1Da_7 protein/kg, the highest dosage level tested. The no-mortality level and the no-observed-adverse-effect level (NOAEL) were 1000 mg Cry1Da_7 protein/kg bw.

J.27. Evaluation of the Potential Effects of Cry1Da_7 Protein on the Mouse, *Mus musculus*

Materials and Methods

Materials

Test material and test dose solution:	
Test item:	Cry1Da_7 protein
Description:	Light brown liquid
Purity corrected concentration:	80.8 mg/mL
Control:	Bovine serum albumin (BSA)
Vehicle control:	10 mM sodium carbonate/bicarbonate, 5 mM cysteine pH 10 with the permeate collected during concentration of the test substance
Test organisms:	
Species:	CD-1 mice (*Mus musculus*)
Age:	8 weeks
Sex:	10 males/10 females per treatment group
Weight:	32.8-39.1 g males/ 24.8-33.1 g females
Diet/Food:	PMI Nutrition International Certified Rodent Chow No. 5002, *ad libitum*
Acclimation Period:	7 days
Environmental conditions:	
Temperature:	21-22°C
Humidity:	47-57%
Photoperiod:	12 hours light / 12 hours dark

Study Design and Methods

Experimental treatments: After an acclimation period of 7 days in their designated housing, ten male and ten female mice were indiscriminately assigned to the treatment and control groups. Dosages used in this study were 0 mg of vehicle dose substance (VDS) per kg, 5000 mg of control (BSA) dose substance (CDS) per kg and 5000 mg of test (MON 95379) dose substance (TDS) per kg. Any assigned animal considered unsuitable for use in the study was replaced by an alternate animal obtained from the same shipment and maintained under the same environmental conditions. The animals were housed individually throughout the study in polycarbonate cages containing appropriate bedding equipped with an automatic watering valve. Animals were provided with enrichment items and edible treats for psychological/environmental enrichment, except when interrupted by study procedures/activities. On Day 0, the animals chosen for use on study were weighed and fasted approximately 3 to 4 hours prior to dose administration. The test, control, and vehicle dosing solutions were administered in 2 doses (approximately 3 to 4 hours apart), each with a dose volume of 33.3 mL/kg body weight by oral gavage on Day 0 to the appropriate group

of 10 males and 10 females. Individual doses were calculated based on the animal's non-fasted (Day 0) body weight. The animals were fasted between doses and were returned to feed following the second dose.

Observations: After dosing, the animals were observed for general health/mortality and moribundity twice daily, once in the morning and afternoon, throughout the study. The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, and gross necropsy findings. Each animal was observed for clinical signs once prior to study start, once prior to dosing on Day 0, a minimum of 2 times postdose on Day 0 (1 within 1 hour of the first dose and 1 approximately 2 hours following the second dose), and daily thereafter (Days 1 to 14). A final detailed clinical observation was performed for each animal on the day of scheduled euthanasia (Day 14). Body weights were measured individually once prior to study start (Day -5), prior to fasting (Day 0), prior to dosing (Day 0, fasted), and on Days 7 and 14 of the study. Feed consumption was determined for each animal on Days 0, 7, and 14 and was reported for the intervals from Study Days 0 to 7 and 7 to 14. At study termination, three birds from each group were subjected to a gross necropsy.

Statistical calculations: Body weight, body weight change and food consumption were compared between the three treatment groups using an overall one-way ANOVA F-test or Kruskal-Wallis test (if parametric assumptions were not met) at the 5% significance level, but excluded semi-quantitative data, and any group with less than 3 observations. Levene's test was used to assess the homogeneity of group variances parametric assumption at the 5% significance level. Pairwise comparisons were conducted between TDS and the control groups using a two-sided Dunnett's or Dunn's test, respectively, if the overall test was significant. All significant pairwise comparisons were reported at the 0.1%, 1%, and 5% significance levels.

Results

There were no test substance-related on survival or clinical signs during the study, except one female in the CDS group that was considered incidental and not CDS related as the cause of death was not determined.

There were no significant differences noted in mean body weights, body weight gains or food consumption for males. There were significant decreases in mean bw and mean bw gain for the TDS females compared to VDS females for Study Days 7 and 14, and Days 0 to 14, respectively. Since the mean body weight and mean body weight gain values were within the historical control data range, these differences were not considered adverse. Food consumption for TDS females when compared to VDS females was significantly decreased for Study Days 0 to 7. However, this was a transient decrease as food consumption was not decreased for Study Days 7 to 14. In addition, the mean food consumption value for the females from Days 0 to 7 were within the historical control data range for both VDS and CDS females. Therefore, due to the variability in

food consumption values for mice, this difference was considered incidental and not treatment related.

There were no test substance-related effects on gross necropsy findings. A single finding of an ovarian cyst was noted on a TDS female, however, this lesion has been noted in control groups in previous studies at an incidence level of 10-20%. Therefore, this finding was considered incidental and not treatment related.

Assay Validity Criteria

The analytical tests established stability and homogeneity of the test and control dosing solutions administered in the mouse oral acute gavage toxicity with Cry1Da_7 protein.

Conclusion

There were no test substance-related adverse effects of Cry1Da_7 protein when administered by oral gavage at a limit dose of 5000 mg/kg body weight in male and female CD-1 mice on mortality, clinical signs, body weights, body weight gains, food consumption or gross pathology findings. Therefore, the NOAEL for Cry1Da_7 protein was 5000 mg/kg body weight.

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Appendix K: Read Mappings and Associated Metrics for all Test, Control, and Reference Substances in the Molecular Characterization of MON 95379

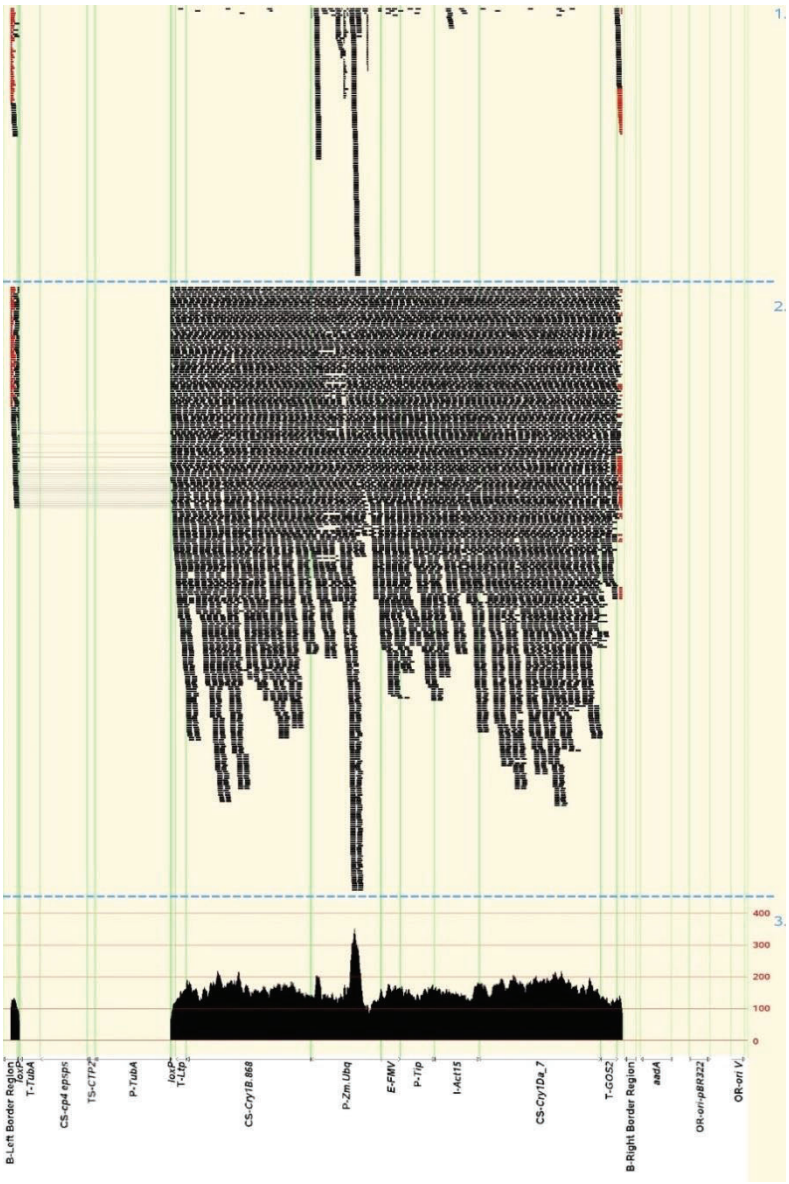


Figure K-1. Read Mapping of MON 95379 (F5) Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines show genetic element boundaries. The region of flank junction sequences that align with the transformation plasmid are shown in red.

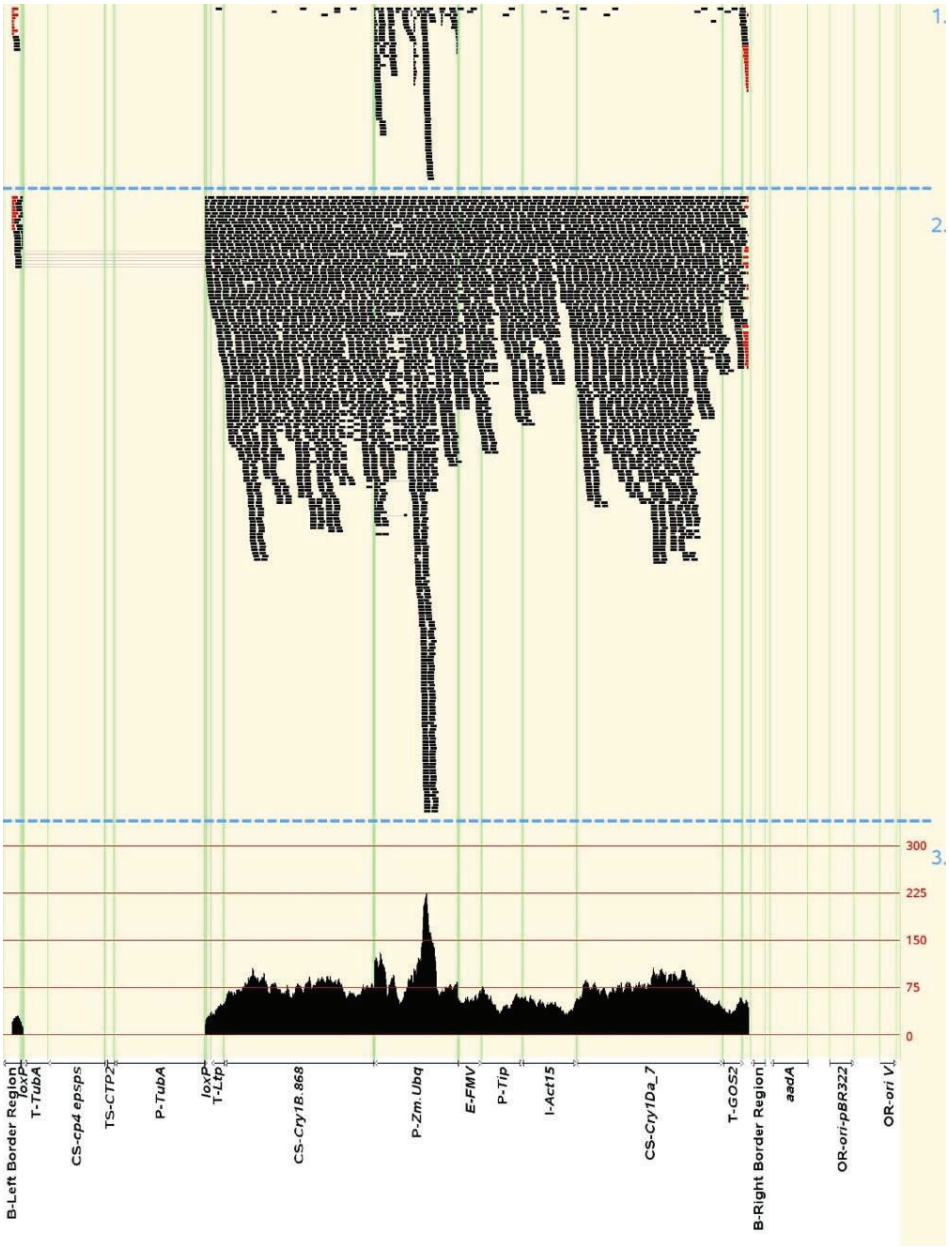


Figure K-2. Read Mapping of MON 95379 (F4F1) Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines show genetic element boundaries. The region of flank junction sequences that align with the transformation plasmid are shown in red.

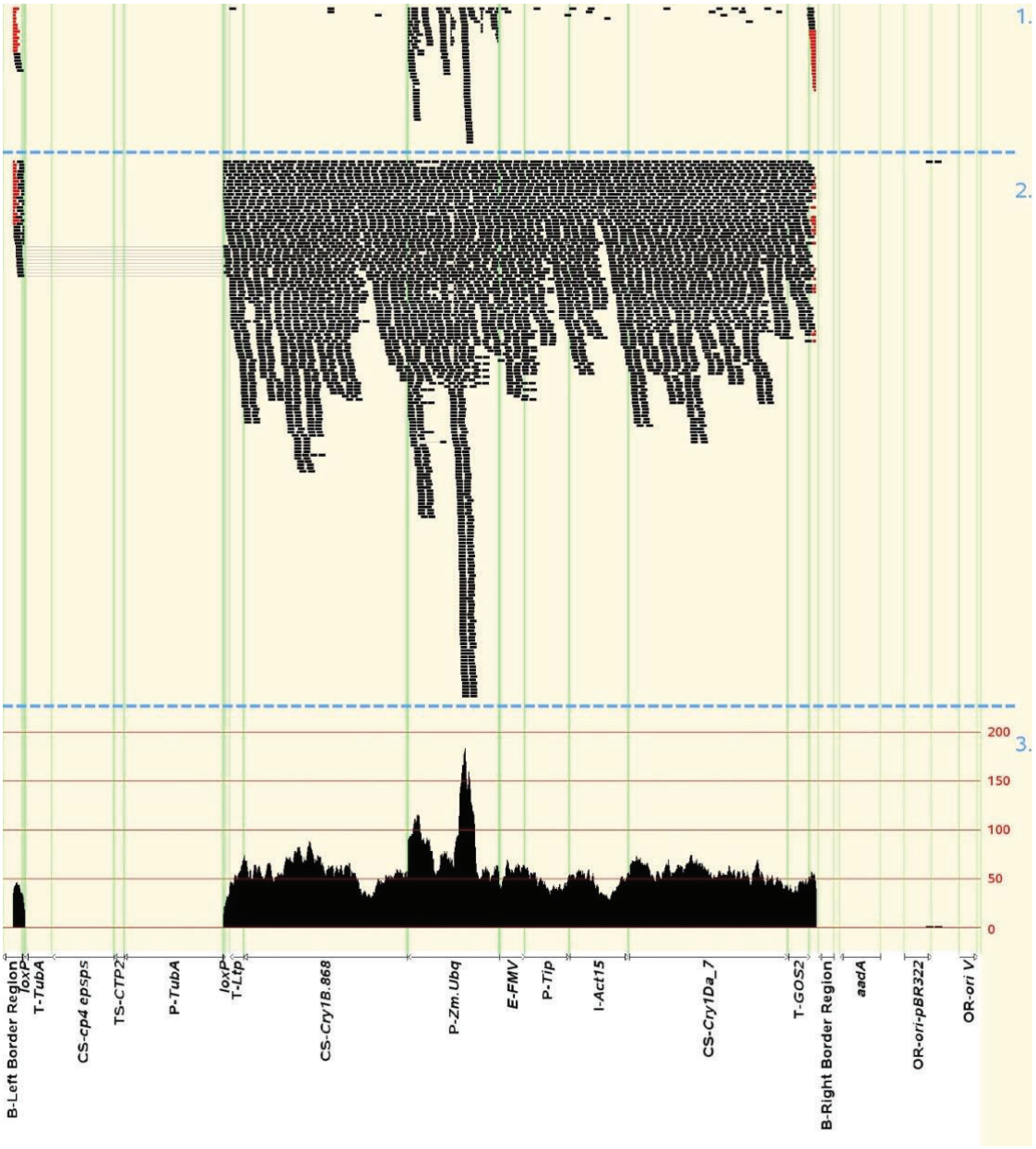


Figure K-3. Read Mapping of MON 95379 (F5F1) Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines show genetic element boundaries. The region of flank junction sequences that align with the transformation plasmid are shown in red.

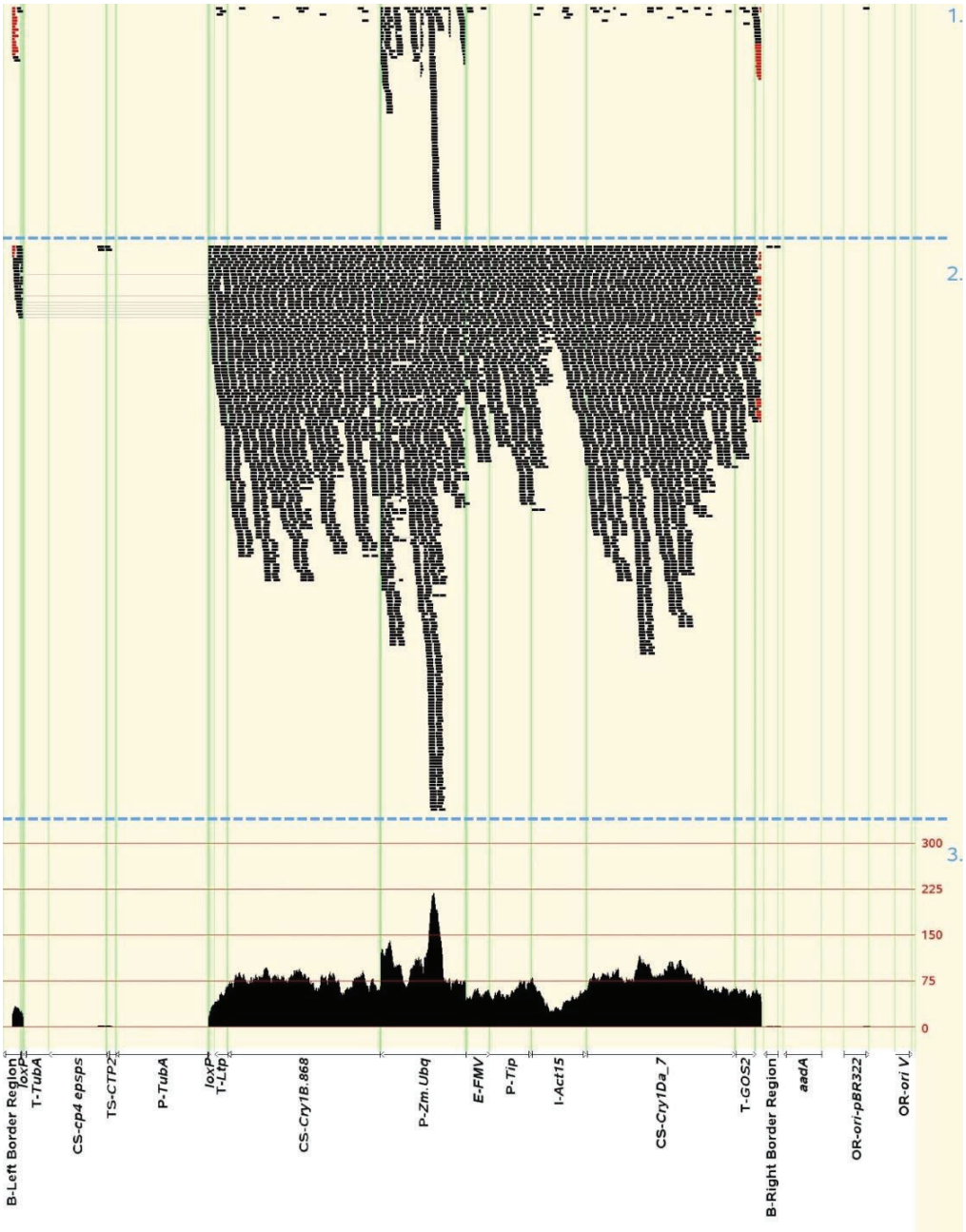


Figure K-4. Read Mapping of MON 95379 (F6F1) Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines show genetic element boundaries. The region of flank junction sequences that align with the transformation plasmid are shown in red.

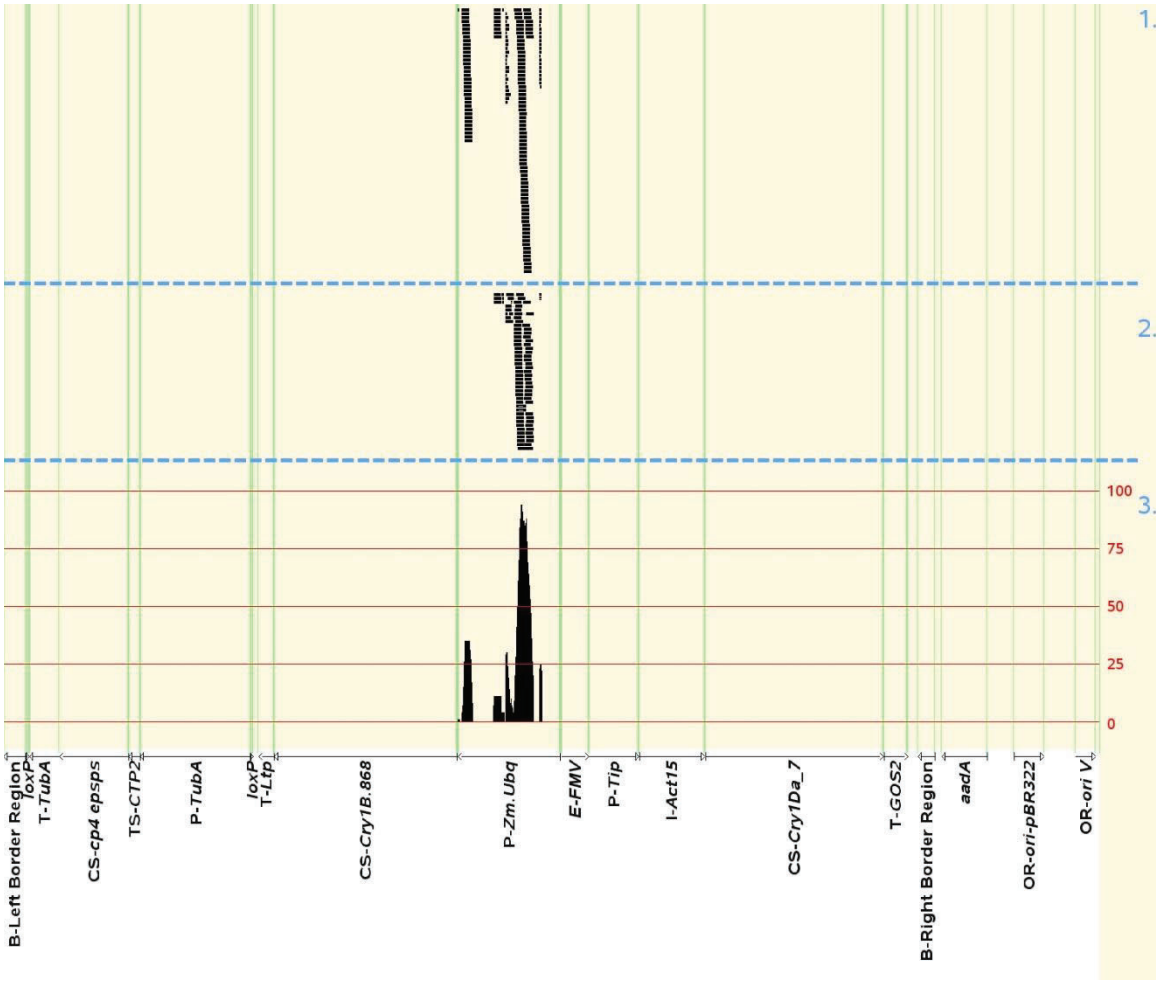


Figure K-5. Read Mapping of Conventional Maize LH244 Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines show genetic element boundaries.

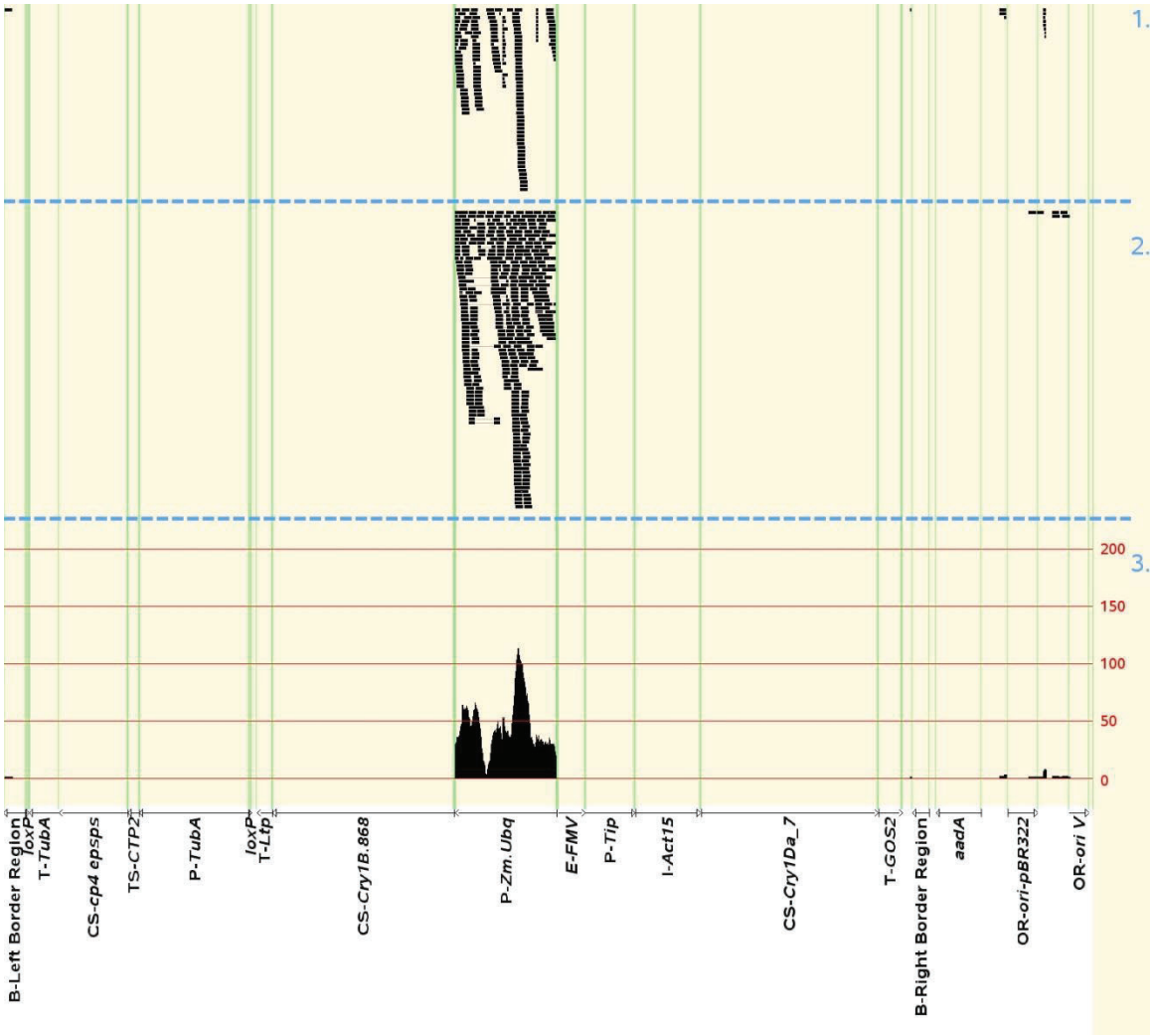


Figure K-6. Read Mapping of Conventional Maize LH244 × HCL617 Versus PV-ZMIR522223

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines show genetic element boundaries.

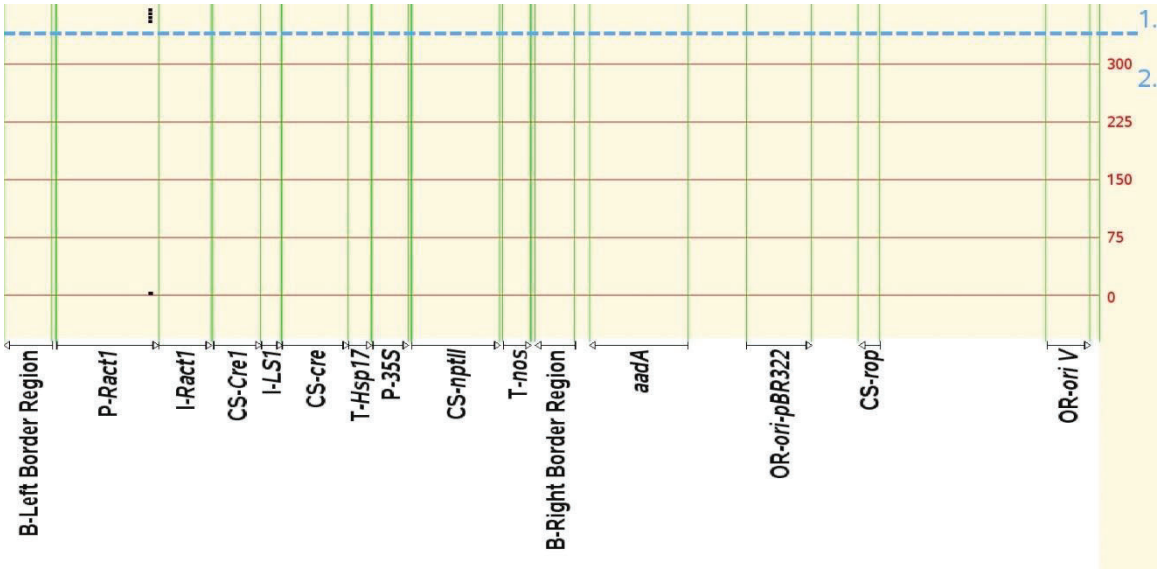


Figure K-7. Read Mapping of Conventional Maize LH244 Versus PV-ZMOO513642

Panel 1 shows the location of unpaired mapped reads, there are no paired mapped reads, and Panel 2 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines show genetic element boundaries.

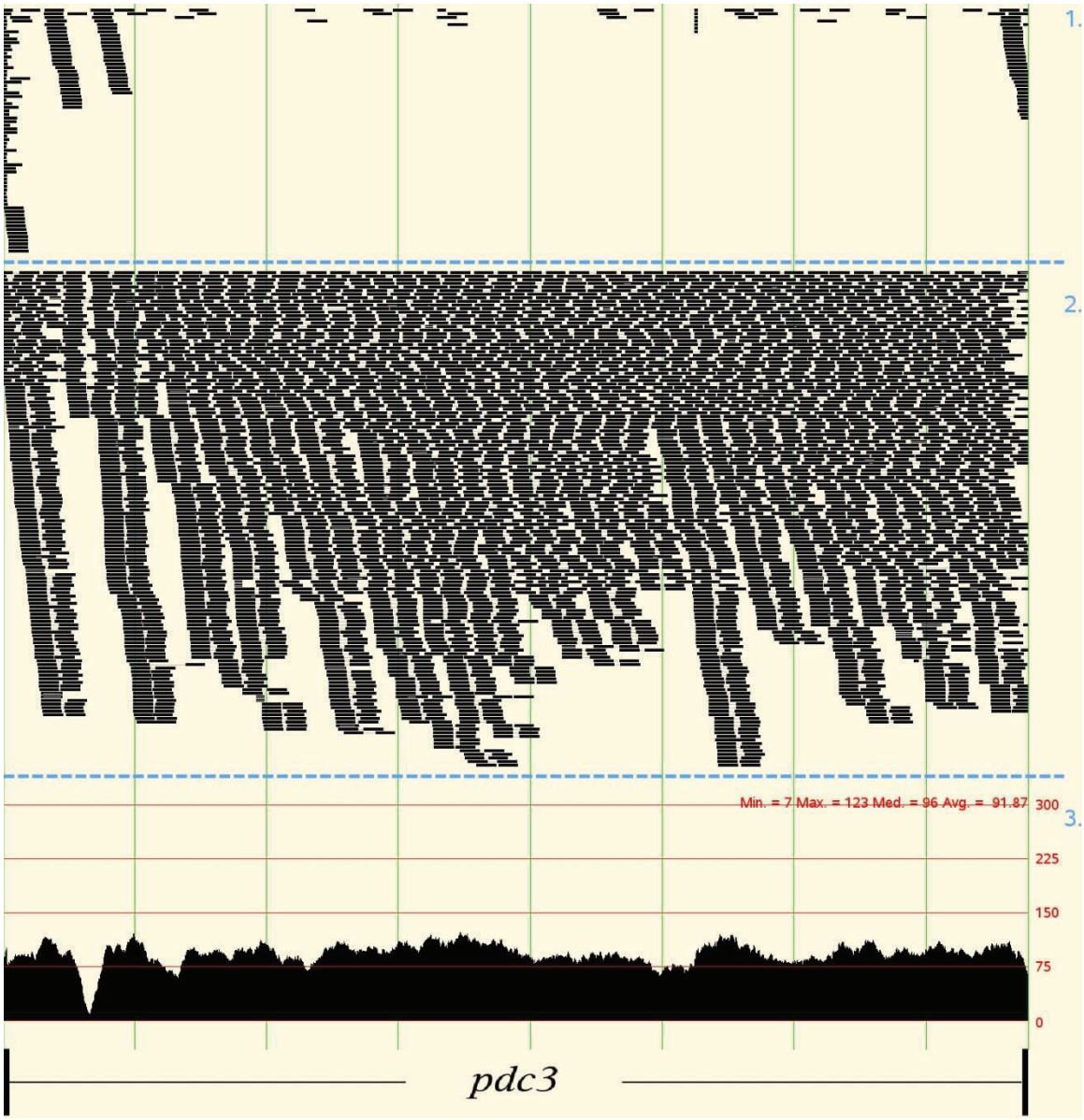


Figure K-8. Read Mapping of MON 95379 (F4) Versus *pdc3*

Panel 1 shows the location of reads that are unpaired when mapped, Panel 2 shows reads that are paired when mapped and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines represent 1000 nucleotide intervals.

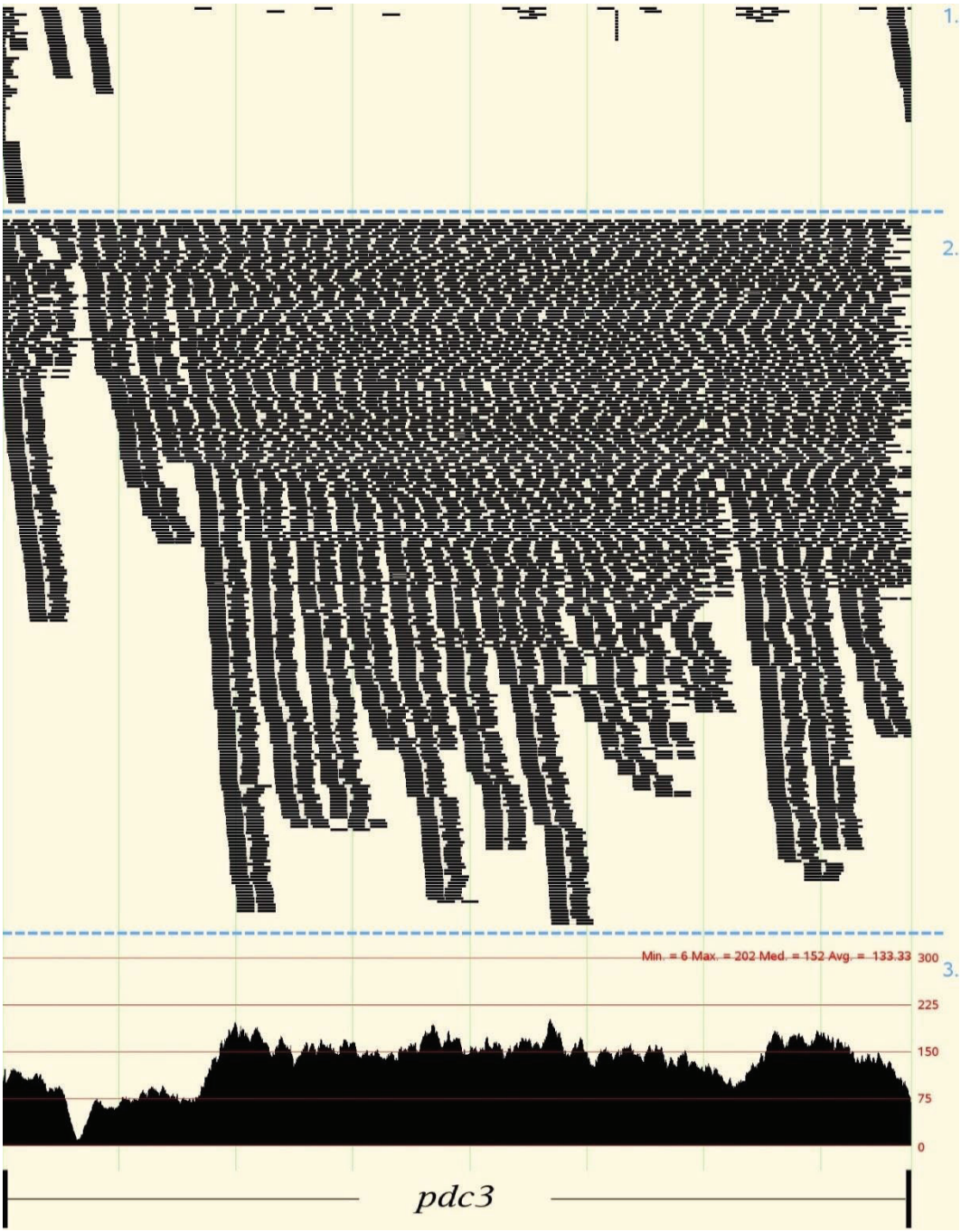


Figure K-9. Read Mapping of MON 95379 (F5) Versus *pdc3*

Panel 1 shows the location of reads that are unpaired when mapped, Panel 2 shows reads that are paired when mapped and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines represent 1000 nucleotide intervals.

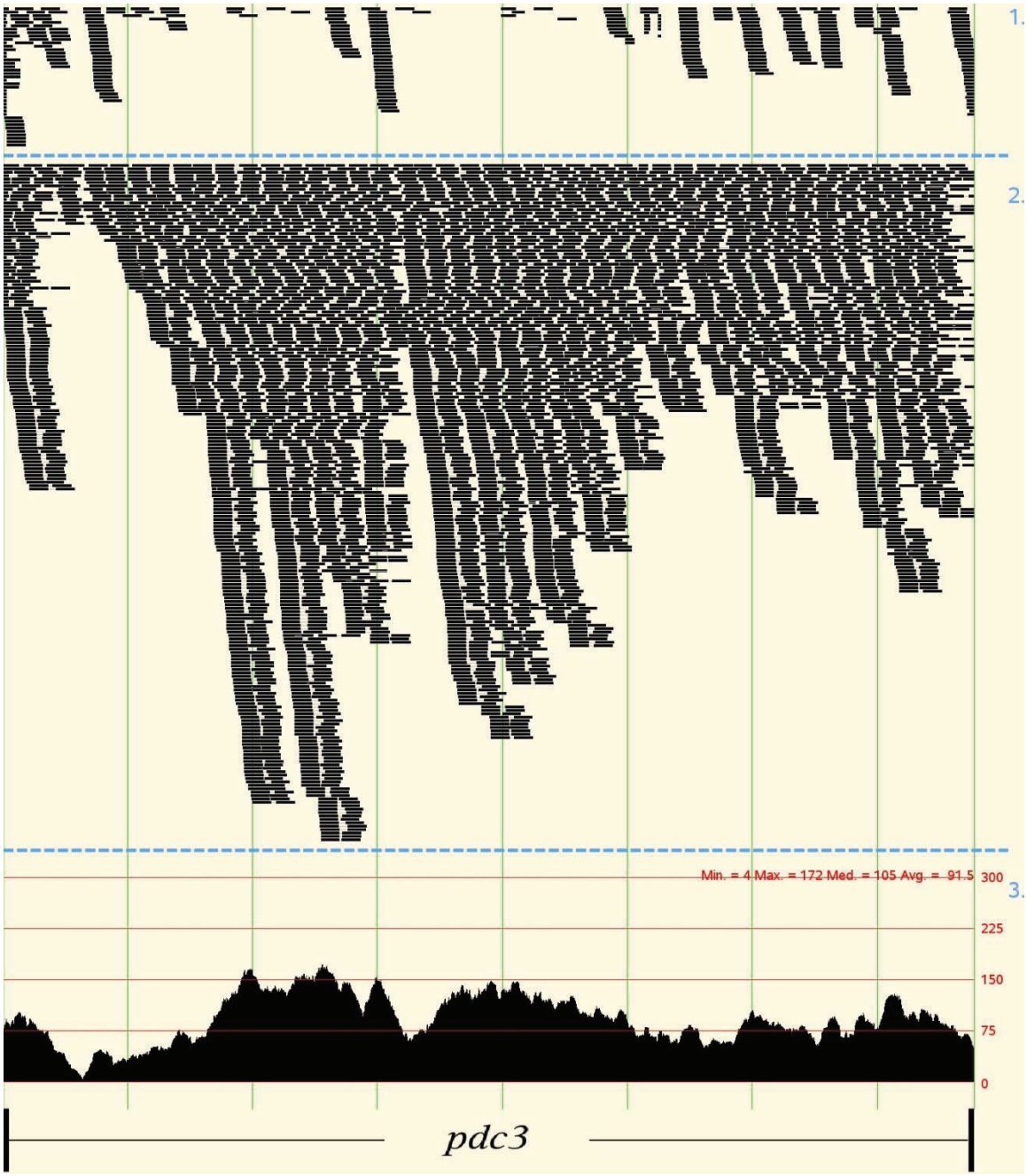
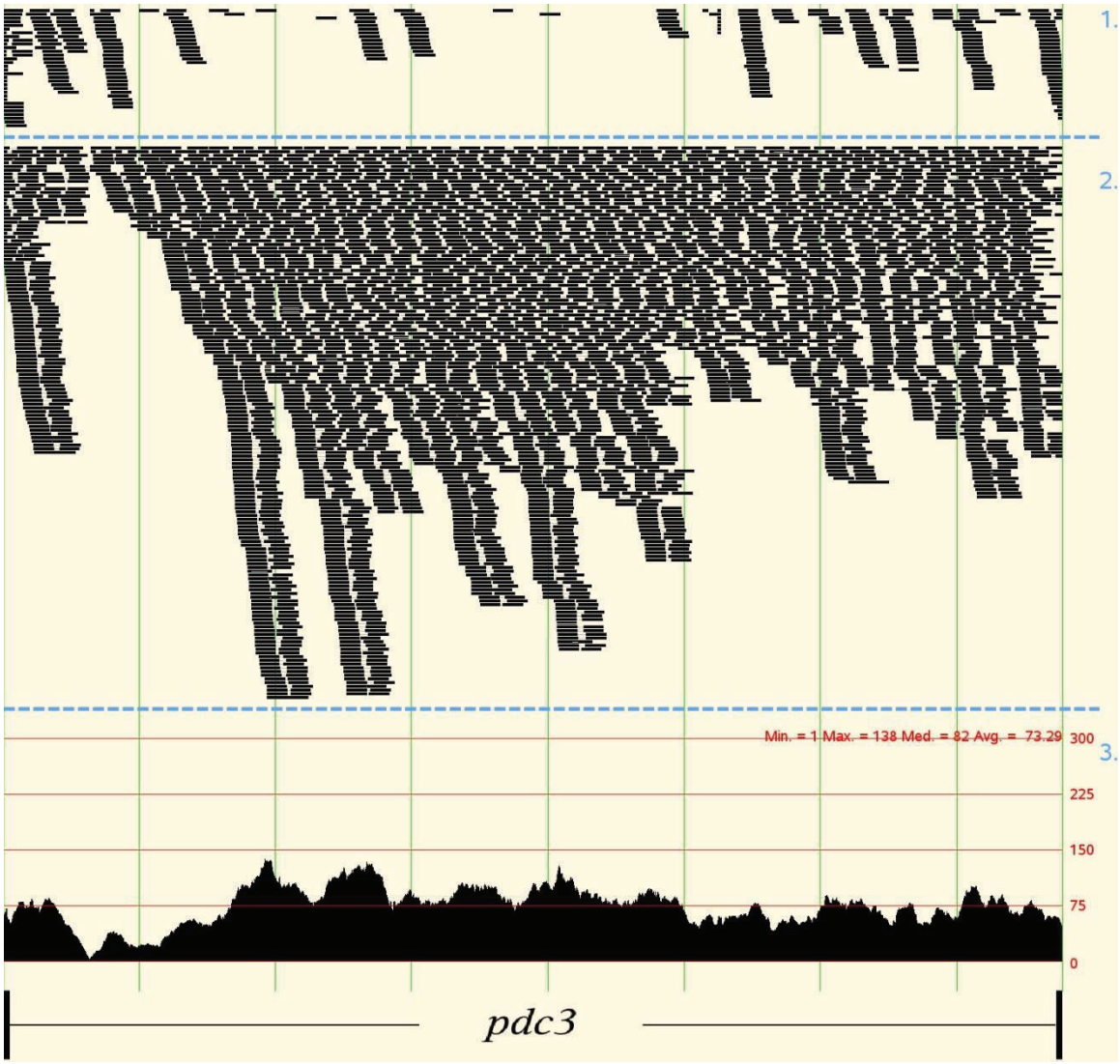


Figure K-10. Read Mapping of MON 95379 (F4F1) Versus *pdc3*

Panel 1 shows the location of reads that are unpaired when mapped, Panel 2 shows reads that are paired when mapped and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines represent 1000 nucleotide intervals.



Appendix Figure K11. Read Mapping of MON 95379 (F5F1) Versus *pdc3*

Panel 1 shows the location of reads that are unpaired when mapped, Panel 2 shows reads that are paired when mapped and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines represent 1000 nucleotide intervals.

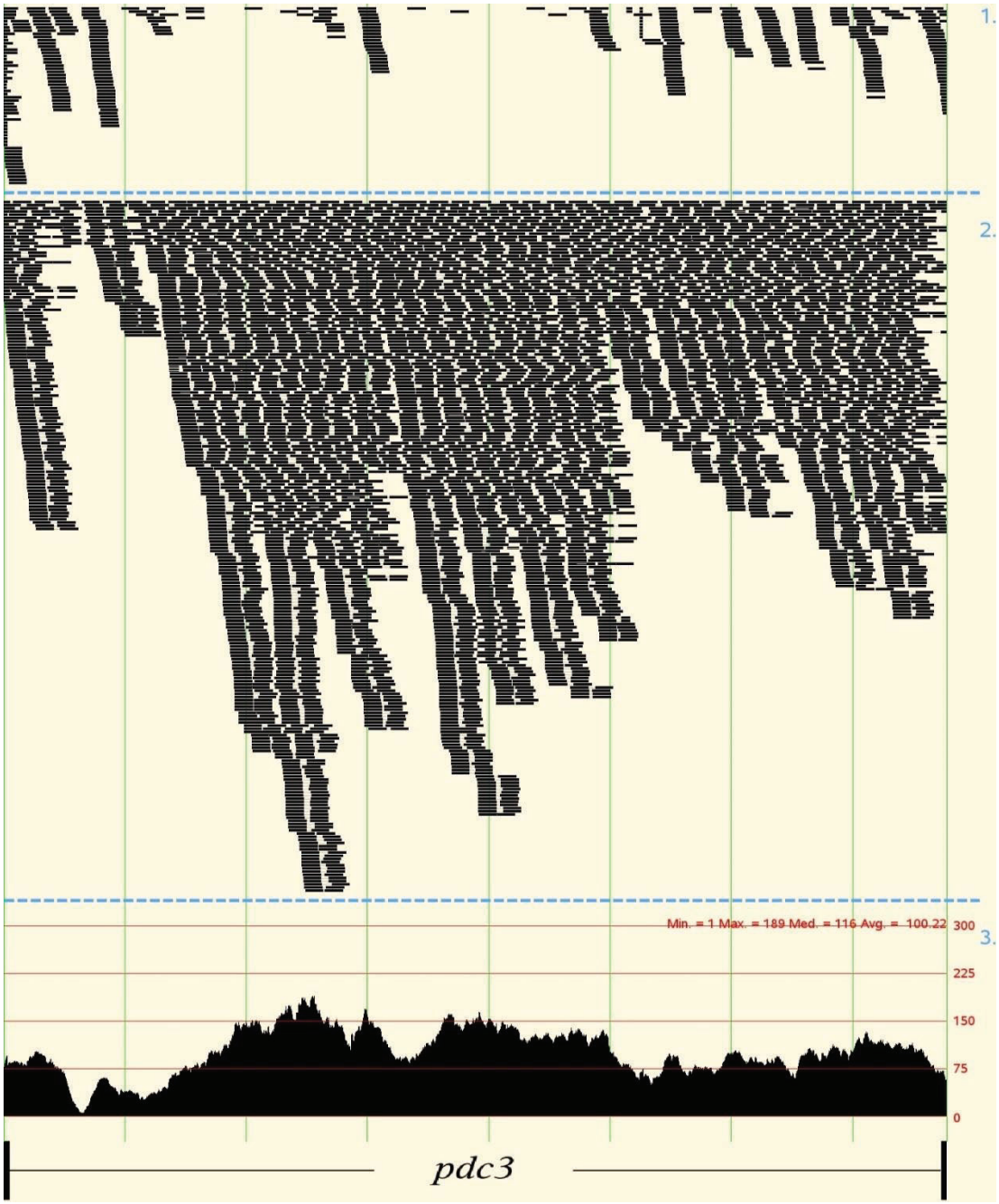


Figure K-12. Read Mapping of MON 95379 (F6F1) Versus *pdc3*

Panel 1 shows the location of reads that are unpaired when mapped, Panel 2 shows reads that are paired when mapped and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines represent 1000 nucleotide intervals.

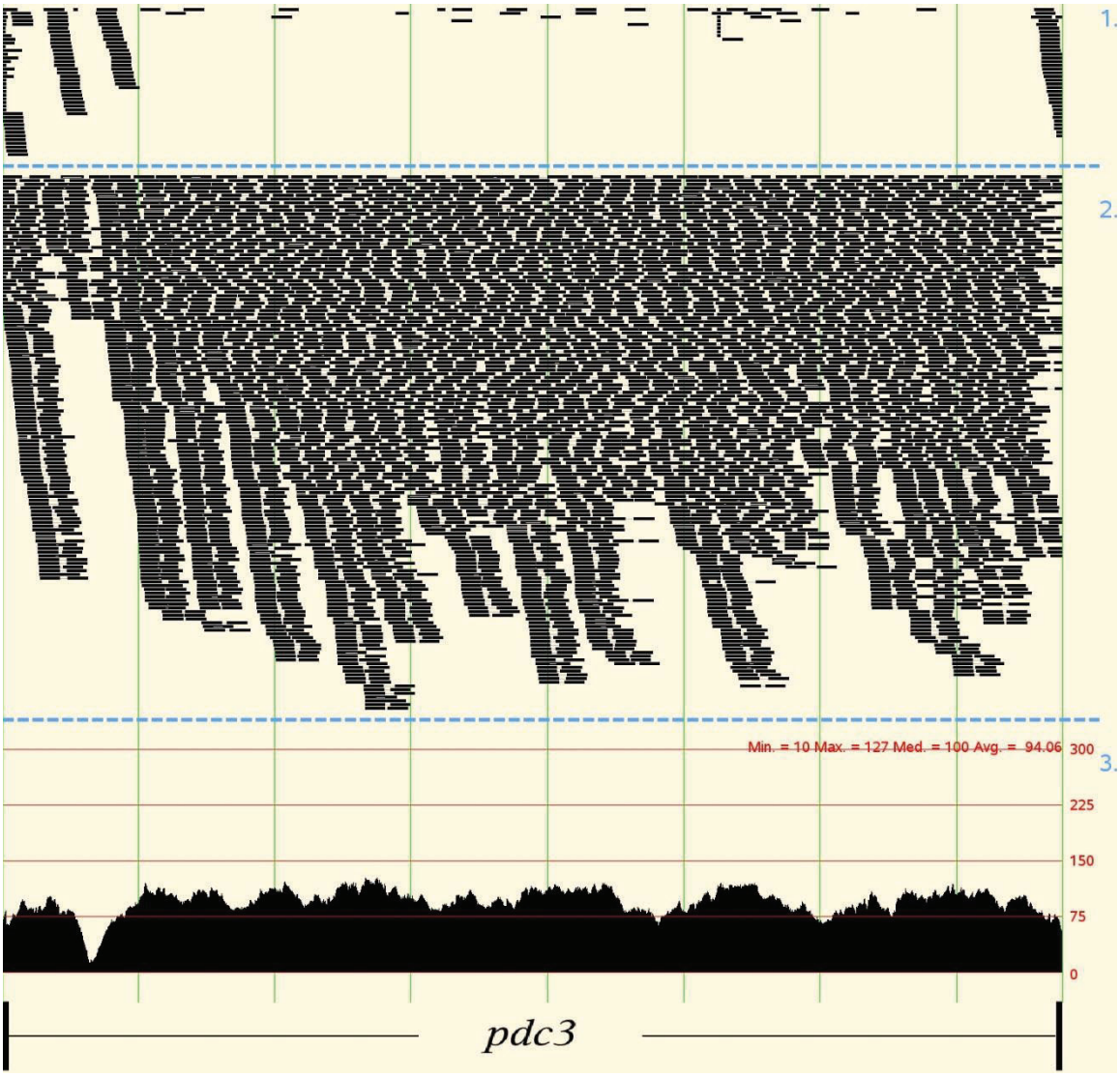


Figure K-13. Read Mapping of LH244 Conventional Maize Versus *pdc3*

Panel 1 shows the location of reads that are unpaired when mapped, Panel 2 shows reads that are paired when mapped and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines represent 1000 nucleotide intervals.

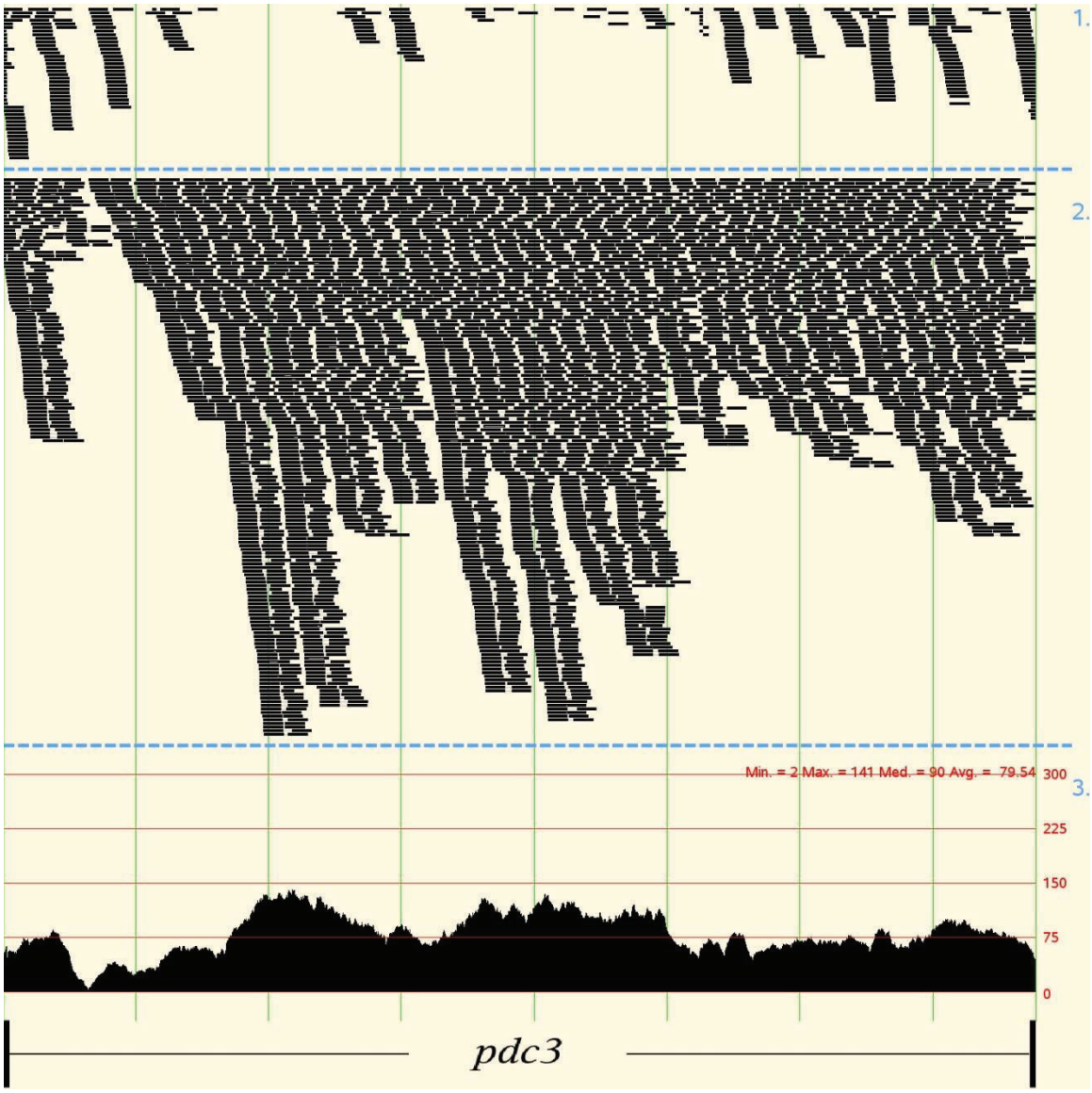


Figure K-14. Read Mapping of LH244 × HCL617 Conventional Maize Versus *pdc3*

Panel 1 shows the location of reads that are unpaired when mapped, Panel 2 shows reads that are paired when mapped and Panel 3 shows a representation of combined raw read depth for unpaired and paired reads across the reference. Vertical lines represent 1000 nucleotide intervals.

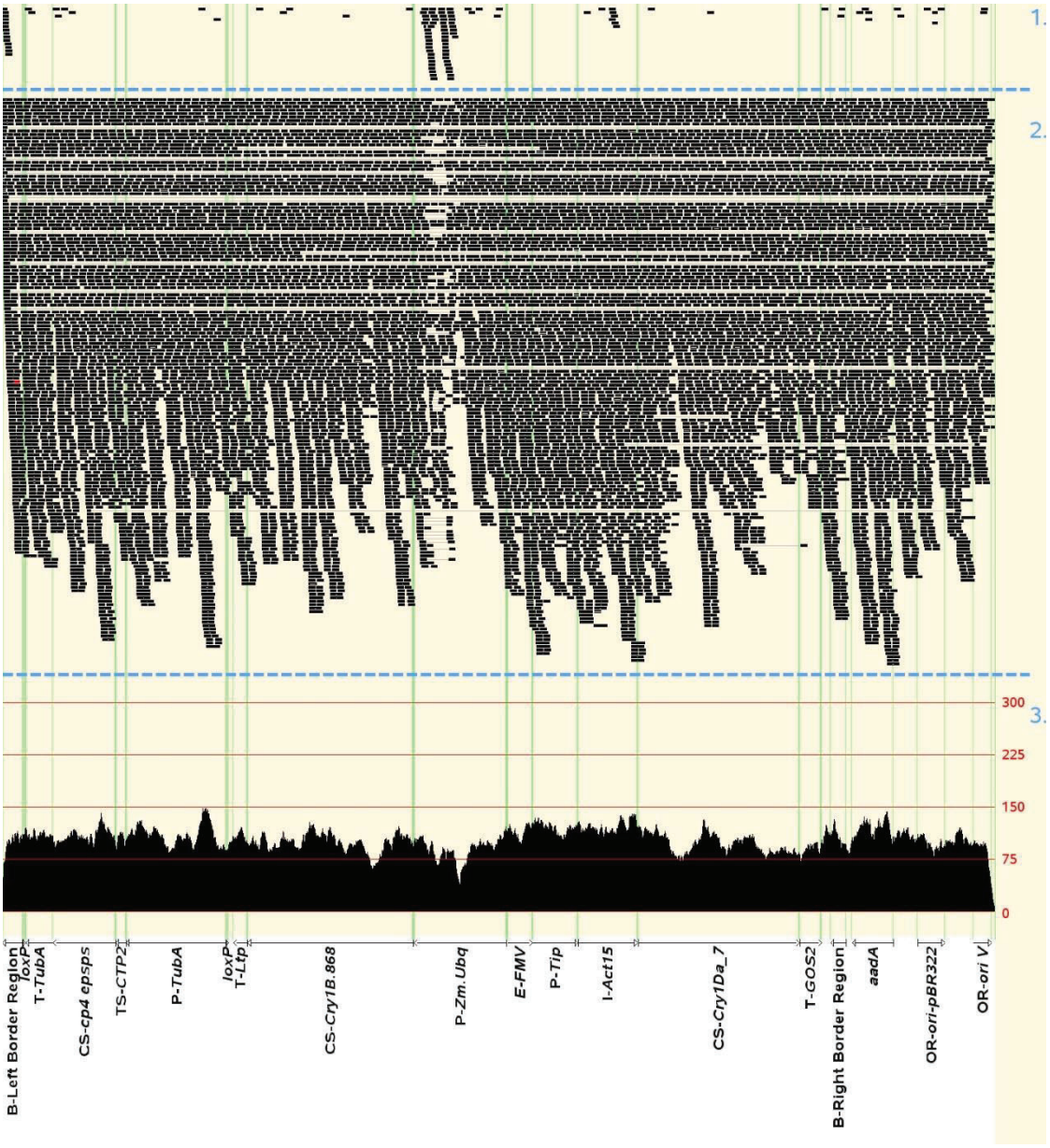


Figure K-15. Read Mapping of the PV-ZMIR522223 NGS Library (1 genome equivalent)

PV-ZMIR522223 sequence reads mapping to the plasmid reference sequence were randomly sampled to yield a 1 genome equivalent number of reads. Panel 1 shows the location of reads that are unpaired when mapped. Panel 2 shows reads that are paired when mapped. Panel 3 shows a representation of combined raw read depth for all reads. Vertical lines show genetic element boundaries.

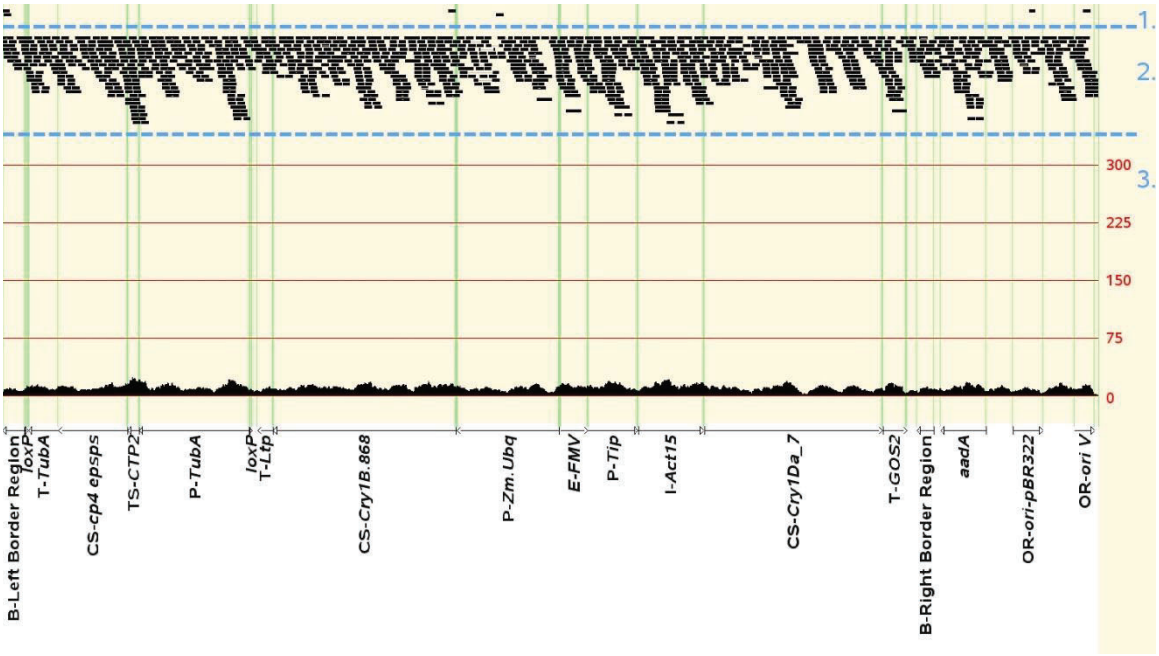


Figure K-16. Read Mapping of the PV-ZMIR522223 NGS Library (1/10th genome equivalent)

PV-ZMIR522223 sequence reads mapping to the plasmid reference sequence were randomly sampled to yield a 1/10th genome equivalent number of reads. Panel 1 shows the location of reads that are unpaired when mapped. Panel 2 shows reads that are paired when mapped. Panel 3 shows a representation of combined raw read depth for all reads. Vertical lines show genetic element boundaries.

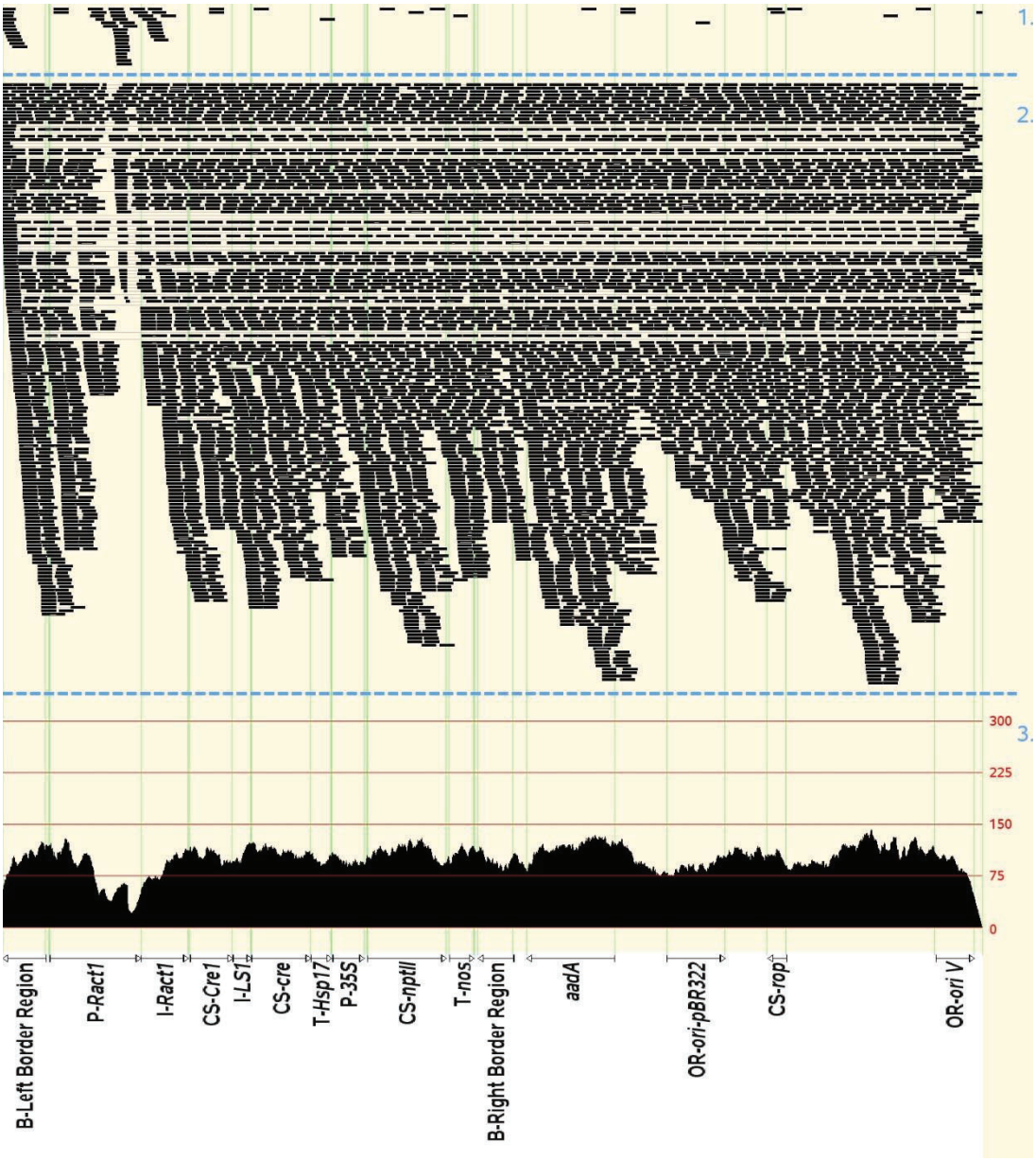


Figure K-17. Read Mapping of the PV-ZMOO513642 NGS Library (1 genome equivalent)

PV-ZMOO513642 sequence reads mapping to the plasmid reference sequence were randomly sampled to yield a 1 genome equivalent number of reads. Panel 1 shows the location of reads that are unpaired when mapped. Panel 2 shows reads that are paired when mapped. Panel 3 shows a representation of combined raw read depth for all reads. Vertical lines show genetic element boundaries.

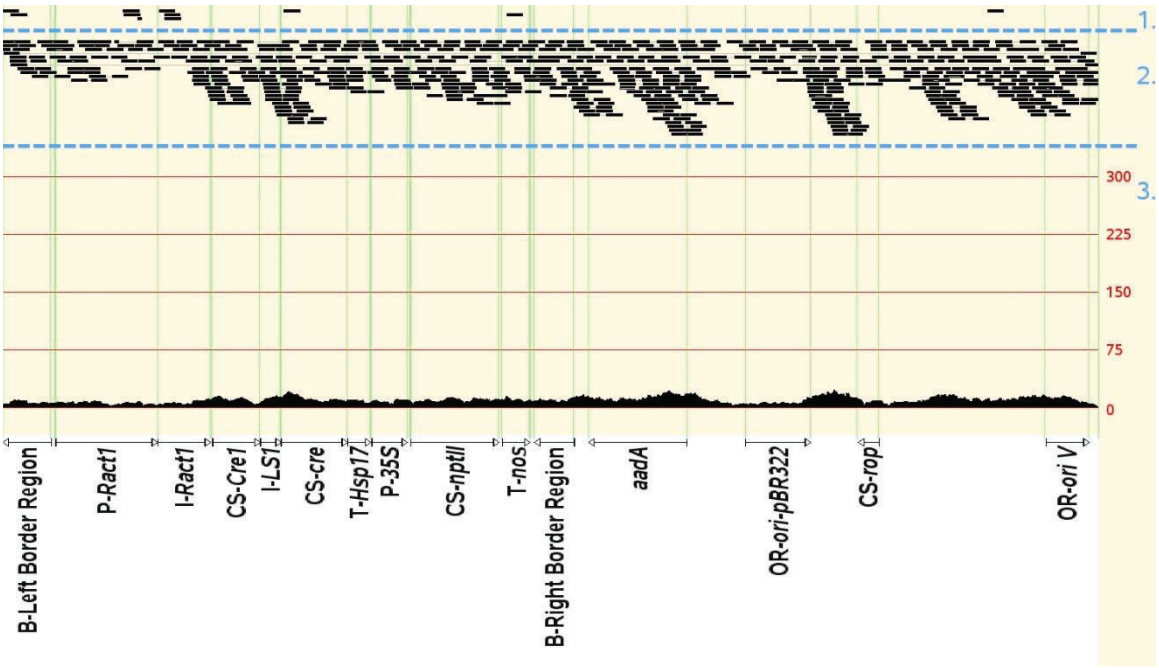


Figure K-18. Read Mapping of the PV-ZMOO513642 NGS Library (1/10th genome equivalent)

PV-ZMOO513642 sequence reads mapping to the plasmid reference sequence were randomly sampled to yield a 1/10th genome equivalent number of reads. Panel 1 shows the location of reads that are unpaired when mapped. Panel 2 shows reads that are paired when mapped. Panel 3 shows a representation of combined raw read depth for all reads. Vertical lines show genetic element boundaries.

Junction A Alignment (All Generations)

```
Directed_Seq_5p  GGCCGGCGCCTGATCGACTTCTCTTCCGCCGCCTTCGCTAGCTATAGCTTGGTAGTAGTC
F4               -----------GATCGACTTCTCTTCCGCCGCCTTCGCTAGCTATAGCTTGGTAGTAGTC
F5               -----------GATCGACTTCTCTTCCGCCGCCTTCGCTAGCTATAGCTTGGTAGTAGTC
F4F1             --------------CGACTTCTCTTCCGCCGCCTTCGCTAGCTATAGCTTGGTAGTAGTC
F5F1             ----------------------------------TCGCTAGCTATAGCTTGGTAGTAGTC
F6F1             -------------------------------------AGCTATAGCTTGGTAGTAGTC

Directed_Seq_5p  ACCAACAACGACCTAGGGAGCTAGCGTACGTTCGAGGTACCTGTCCTGCAAGCAAATGTG
F4               ACCAACAACGACCTAGGGAGCTAGCGTACGTTCGAGGTACCTGTCCTGCAAGCAAATGTG
F5               ACCAACAACGACCTAGGGAGCTAGCGTACGTTCGAGGTACCTGTCCTGCAAGCAAATGTG
F4F1             ACCAACAACGACCTAGGGAGCTAGCGTACGTTCGAGGTACCTGTCCTGCAAGCAAATGTG
F5F1             ACCAACAACGACCTAGGGAGCTAGCGTACGTTCGAGGTACCTGTCCTGCAAGCAAATGTG
F6F1             ACCAACAACGACCTAGGGAGCTAGCGTACGTTCGAGGTACCTGTCCTGCAAGCAAATGTG

Directed_Seq_5p  TGGTAGTGGT^CCAATTTTTTTTCAATTCAAAAATGTAGATGTCCGCAGCGTTATTATAA
F4               TGGTAGTGGT^CCAATTTTTTTTCAATTCAAAAATGTAGATGTCCGCAGCGTTATTATAA
F5               TGGTAGTGGT^CCAATTTTTTTTCAATTCAAAAATGTAGATGTCCGCAGCGTTATTATAA
F4F1             TGGTAGTGGT^CCAATTTTTTTTCAATTCAAAAATGTAGATGTCCGCAGCGTTATTATAA
F5F1             TGGTAGTGGT^CCAATTTTTTTTCAATTCAAAAATGTAGATGTCCGCAGCGTTATTATAA
F6F1             TGGTAGTGGT^CCAATTTTTTTTCAATTCAAAAATGTAGATGTCCGCAGCGTTATTATAA

Directed_Seq_5p  AATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGTATTTATAGGCGAAAGC
F4               AATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGTATTTATAGGCGAAAGC
F5               AATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGTATTTATAGGCGAAAGC
F4F1             AATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGTATTTATAGGCGAAAGC
F5F1             AATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGTATTTATAGGCGAAAGC
F6F1             AATGAAAGTACATTTTGATAAAACGACAAATTACGATCCGTCGTATTTATAGGCGAAAGC

Directed_Seq_5p  AATAAACAAATTATTCTAATTCGGAAATCTTTATTTCGACGTGTCTACATTCAC
F4               AATAAACAAATTATTCTAATTCGGAAATCTTTATTTCGACGT------------
F5               AATAAACAAATTATTCTAATTCGGAAATCTTTATTTC-----------------
F4F1             AATAAACAAATTATTCTAATTCGGAAATCTTTA--------------------
F5F1             AATAAACAAATTATTCTAATTCGGAAATCTTTATTTCGAC--------------
F6F1             AATAAACAAATTATTCTAATTCGGAAATCTTTATTTC-----------------
```

Junction B Alignment (All Generations)

```
Directed_Seq_3p  GCTTCAGTTAATTAAAAGTAAGGGTCCCAAGTAAGGCCGGCCAAGTAACGGTCCGAAGTA
F4               ----------ATTAAAAGTAAGGGTCCCAAGTAAGGCCGGCCAAGTAACGGTCCGAAGTA
F5               -----------TTAAAAGTAAGGGTCCCAAGTAAGGCCGGCCAAGTAACGGTCCGAAGTA
F4F1             -----------TTAAAAGTAAGGGTCCCAAGTAAGGCCGGCCAAGTAACGGTCCGAAGTA
F5F1             ---------------AAGTAAGGGTCCCAAGTAAGGCCGGCCAAGTAACGGTCCGAAGTA
F6F1             ------------TAAAAGTAAGGGTCCCAAGTAAGGCCGGCCAAGTAACGGTCCGAAGTA
```

```
Directed_Seq_3p  AGGCGCGGGTACCGTCGGTCCGGGCCTAGTAGGCCAAGCAGGACGTGGCGCGCCAAGAAG
F4               AGGCGCGGGTACCGTCGGTCCGGGCCTAGTAGGCCAAGCAGGACGTGGCGCGCCAAGAAG
F5               AGGCGCGGGTACCGTCGGTCCGGGCCTAGTAGGCCAAGCAGGACGTGGCGCGCCAAGAAG
F4F1             AGGCGCGGGTACCGTCGGTCCGGGCCTAGTAGGCCAAGCAGGACGTGGCGCGCCAAGAAG
F5F1             AGGCGCGGGTACCGTCGGTCCGGGCCTAGTAGGCCAAGCAGGACGTGGCGCGCCAAGAAG
F6F1             AGGCGCGGGTACCGTCGGTCCGGGCCTAGTAGGCCAAGCAGGACGTGGCGCGCCAAGAAG

Directed_Seq_3p  AACGATTGGCAAACAGCTATTATGGGTATTATGGGTAGGC^ACATGGGAATATAGTGGGA
F4               AACGATTGGCAAACAGCTATTATGGGTATTATGGGTAGGC^ACATGGGAATATAGTGGGA
F5               AACGATTGGCAAACAGCTATTATGGGTATTATGGGTAGGC^ACATGGGAATATAGTGGGA
F4F1             AACGATTGGCAAACAGCTATTATGGGTATTATGGGTAGGC^ACATGGGAATATAGTGGGA
F5F1             AACGATTGGCAAACAGCTATTATGGGTATTATGGGTAGGC^ACATGGGAATATAGTGGGA
F6F1             AACGATTGGCAAACAGCTATTATGGGTATTATGGGTAGGC^ACATGGGAATATAGTGGGA

Directed_Seq_3p  GGCAGAGGCGTGCCTGTGCCTGCTCTTAATTTGAGCTCCTCCCCTGGCCCTGATAGGGCA
F4               GGCAGAGGCGTGCCTGTGCCTGCTCTTAATTTGAGCTCCTCCCCTGGCCCTGATAGGGCA
F5               GGCAGAGGCGTGCCTGTGCCTGCTCTTAATTTGAGCTCCTCCCCTGGCCCTGATAGGGCA
F4F1             GGCAGAGGCGTGCCTGTGCCTGCTCTTAATTTGAGCTCCTCCCCTGGCCCTGATAGGGCA
F5F1             GGCAGAGGCGTGCCTGTGCCTGCTCTTAATTTGAGCTCCTCCCCTGGCCCTGATAGGGCA
F6F1             GGCAGAGGCGTGCCTGTGCCTGCTCTTAATTTGAGCTCCTCCCCTGGCCCTGATAGGGCA

Directed_Seq_3p  TGTGCATGCGCACAATAATCATTGGAGCTGCCTGAATGATTTGCATGCGCTC
F4               TGTGCATGCGCACAATAATCATTGGAGCTGCCTGAATGATTT----------
F5               TGTGCATGCGCACAATAATCATTGGAGCTGCCTG------------------
F4F1             TGTGCATGCGCACAATAATCATTGGAGCTG----------------------
F5F1             TGTGCATGCGCACAATAATCATTGGAGCT-----------------------
F6F1             TGTGCATGCGCACAATAATCATTGGAGCTGCCTGAATGATTT----------
```

Figure K-19. Junction Sequences Detected: All Breeding Generations

Full consensus sequence for Junction A and Junction B showing exact alignment to the independently determined *in planta* locus specific sequence (labeled "Directed_Seq" in the figure), the individual consensus sequences for each of the five generations are labeled according to their generation (F4, F5, F4F1, F5F1, and F6F1). Double underlined text indicates plasmid-derived T-DNA sequence, single underlined text indicates flank sequence, and the caret character "^" indicates the junction point between the insert and the flank. Dash characters "-" indicate positions past the end of the consensus sequence for a particular generation.

Table K-1. Summary of total Generated NGS Data for the Control and Test Substances

Sample	Total Nucleotide Number (Gb)[1]	Hypothetical Genome Coverage (×-fold)[2]	Effective Median Depth of Coverage (×-fold)[3]
LH244	202.18	88×	100×
LH244 × HCL617	246.48	107×	90×
MON 95379 (F4)	191.54	83×	96×
MON 95379 (F5)	314.87	137×	152×
MON 95379 (F4F1)	241.51	105×	105×
MON 95379 (F5F1)	193.37	84×	82×
MON 95379 (F6F1)	247.22	107×	116×

[1] For each sample, the raw data produced are presented in terms of total nucleotide number in gigabases (Gb). Notably, the read lengths generated in this experimate range from 35 to 151bp.

[2] The genome coverage of the sequencing events was estimated based on the *Zea mays* genome sized (approximately 2.3 Gb, Schnable et al., 2009).

[3] Effective depth of coverage is determined by mapping and alignment of all raw data to a single copy locus within the *Zea mays* genome (*pdc3*, GenBank accession AF370006.2). The median effective depth of coverage as aligned against this locus is shown for all samples. The positional raw read depths for all samples can be observed in panel 3 of Appendix Figures K8 through K14.

Table K-2. Summary Coverage Data for the T-DNA region of Control and Test Substances
A summary of the coverage data across the T-DNA insert regions[1] in panel 3 of the mapping figures (Appendix K, Figures K1-K6) for the control libraries and test substance generations.

Sample	Minimum	Maximum	Average (x-fold)	Median (x-fold)
LH244	0	94	2.51×	0×
LH244 × HCL617	0	113	6.7×	0×
MON 95379 (F4)	26	200	94.44×	94×
MON 95379 (F5)	51	351	156.32×	156×
MON 95379 (F4F1)	10	225	68.41×	67×
MON 95379 (F5F1)	13	183	56.59×	53×
MON 95379 (F6F1)	14	218	71.99×	71×

[1]Insert region corresponds to positions 258-514 and 4895-17996 relative to the reference used to generate Figure IV-5, Figure IV-6 and Appendix K Figures K1 through K6.

References for Appendix K

Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton L, Graves TA, Minx P. 2009. The B73 maize genome: complexity, diversity, and dynamics. Science. 326:1112-5.

Appendix L: Supplemental Information

This appendix expands on several areas of the petition, and are provided here for reference.

L.1. Additional Information Regarding Total Quality and Quality of Sequence Data Generated for the Characterization of the Genetic Modification (Section IV)

Notably, in sequencing runs it is possible for a proportion of reads to be returned with a reduced length relative to the targeted sequence length. Although much of the data generated herein is 151 base pairs in length, a portion of the reads is shorter. As such we have reported as the total number of nucleotides in giga-bases (Gb) generated alongside hypothetical coverage base on genome size as it is a directly quantifiable and therefore translative metric regarding total sequencing generated. This data has been provided in Appendix K (Table K-1). Appendix K further includes data summarizing total sequencing generated and coverage metrics for all generations (F4F1, F5, F5F1, and F6F1) and controls (Tables K-1 and K-2), and includes coverage across the T-DNA regions.

The molecular characteristics, including a) the number of inserts, b) the presence or absence of plasmid backbone, and c) the DNA sequence of inserted transformation construct, can be determined using bioinformatics by mapping all reads to the plasmid sequence and selecting reads mapped to the junctions of the insert DNA. Mapping reads back to the entire reference genome is not necessary to determine these desired endpoints of molecular characterization. Furthermore, estimated genome coverage can be calculated using total sequence data generated and supported by the observed coverage of inserted DNA and single copy controls. Data supporting these conclusions are included in the Appendix K, Tables K-1 and K-2.

All reads were mapped to the reference sequences without any exclusion or filtering based on per base quality metrics. However, only mapped reads that meet Bowtie2 mapping criteria (reported in Section B.9.) were displayed in the read mapping figures and used to calculate the effective median coverage against the single copy locus as well as the T-DNA insert coverage. Additionally, reads with partial matches to the plasmid sequence of at least 30 bases and 96.6% or greater identity were classified as potential junction spanning reads and used to determine the immediate T-DNA to flanking genomic DNA sequence at the integration point (reported in Section B.9.1).

L.2. Additional Information Regarding Generations Analyzed and Junction Site Consistency in the Characterization of the DNA Inserts in MON 95379 (Section IV.B)

Five distinct generations were characterized, with those designated with a secondary XXF1 designation representing those crossed with HCL617 making them hybrid generations. A breeding history demonstrating these crosses is provided in Figure IV-4. The total sequencing generated, hypothetical genome coverage, and mapped coverage to the T-DNA for each of the generations are provided in Appendix K (Tables K-1 and K-2).

A consensus of the junction sequences was aligned across all generations alongside an independently sequenced PCR product derived from the same site and is provided in Appendix K

(Figure K-19). The consensus sequences for the two junction sites of the single insert were generated by compiling the corresponding group of junction reads which share the same junction point and common flanking sequence for each of the generations. These sequences are representative of the reads that support the corresponding junction site. The alignment of these sequences demonstrated that they were identical across the entirety of their captured sequence for all samples, supporting the generational stability of the single insert event.

L.3. Additional Information Regarding Consistency of Read Mapping Methodology used in the Characterization of the DNA Inserts in MON 95379 (Section IV.B)

The sequencing herein utilized NGS platforms (Illumina) to conduct whole genome sequencing on multiple generations and controls, as well as transformation plasmids which are described as a "reference substances" to establish the molecular characterization endpoints. Identical library preparation methods, and mapping parameters were used on all samples, including all transformation vectors and controls. Identical software and parameters were used in all mappings generated regardless of the substance/generation characterized, and the mapping parameters used are provided in Section B.9.

L.4. Additional Information Regarding the Endogenous Maize *Zm.Ubq* Element in the Characterization of the DNA Inserts in MON 9539 (Section IV.B)

As discussed in Section IV.B.3 and observed in Figure IV-5, it can be observed that a number of reads align to the *Zm.Ubq* element in the read mapping of the conventional maize LH244 to PV-ZMIR522223. It is important to note that the similarity of endogenous maize *Zm.Ubq* to that present in the MON 95379 T-DNA is unnecessary to establish the endpoints of molecular characterization. Insert copy number is determined by junction site identification and further supported by the relative depth of sequencing, and the use of controls to account for the presence or absence of any other shared feature between the transformed and conventional lines as described in Section IV.B.3. Nevertheless, an alignment of common elements may in some cases provide some additional context as to what is being observed, and is provided in Figure L-1.

Figure L-1. Alignment A. The alignment Between an Endogenous Maize *Zm.Ubq* Sequence and the MON 95379 T-DNA *Zm.Ubq* Element.

```
# fasta36 P-Zm-Ubq.fasta /data/databases/2020/ZMA_2020/GCF_000005005.2_B73_RefGen_v4_genomic.fna
FASTA searches a protein or DNA sequence data bank
version 36.3.5d Aug, 2012(preload8)
Please cite:
W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448
 Query: P-Zm-Ubq.fasta
  1>>>MON95379_P-Zm-Ubq - 2008 nt
Library: /data/databases/2020/ZMA_2020/GCF_000005005.2_B73_RefGen_v4_genomic.fna
  2135083061 residues in   267 sequences
 Statistics:  Expectation_n fit: rho(ln(x))= 18.8188+/- 0.222; mu= -88.5427+/-26.544
mean_var=459.1494+/-454.864, 0's: 0 Z-trim(101.4): 2119  B-trim: 0 in 0/9
```

```
Lambda= 0.059855
statistics sampled from 3448 (5210) to 3448 sequences
Algorithm: FASTA (3.7 Nov 2010) [optimized]
Parameters: +5/-4 matrix (5:-4), open/ext: -12/-4
ktup: 6, E-join: 0.25 (0.412), E-opt: 0.05 (0.248), width:  16
Scan time: 202.060
 The best scores are:                                      opt bits E(14397)
NC_024463.2 Zea mays cultivar B73 chromosome 5 (223902240) [r] 4439 402.6 2.7e-109
 >>NC_024463.2 Zea mays cultivar B73 chromosome 5, B73 Re  (223902240 nt)
rev-comp initn: 5036 init1: 1852 opt: 4439  Z-score: 2069.1  bits: 402.6 E(14397): 2.7e-109
banded Smith-Waterman score: 4989; 86.1% identity (86.1% similar) in 1422 nt overlap (1385-1:84633633-
84635038)
          1410      1400      1390      1380      1370      1360
MON95- CTACTCCGATCTAGAACGACCCTTTATCCTTTAATAAATCCACCAGGTAGTTTGAAACAG
                                      ::::::::::::::::::::::::::::::
NC_024 TATGTATGGCACACACATACAGATCCAAAATTAATAAATCCACCAGGTAGTTTGAAACAG
       84633610  84633620  84633630  84633640  84633650  84633660
          1350      1340      1330      1320      1310      1300
MON95- TATTCTACTCCGATCTAGAACGACCGCCCAACCAGACCATATCATCACAACCAAGCGAGA
        :::::::::::::::::::::::::::::::::::::::: ::::::::::::::::  : :
NC_024 AATTCTACTCCGATCTAGAACGACCGCCCAACCAGACCACATCATCACAACCAAGACA-A
       84633670  84633680  84633690  84633700  84633710  84633720
          1290      1280      1270        1260      1250
MON95- AAAAAAGCATCTCTGTATATGCATCAGTAAAAC--CCGCATCAACATGTAT--ACCTATC
       :::::::::  :    : ::: : : :  ::::    ::: :  ::: :::  :  ::
NC_024 AAAAAAGCA--TGAAAAGATG-ACCCGACAAACAAGTGCA-CGGCATATATTGAAATAAA
        84633730    84633740   84633750   84633760  84633770
    1240      1230      1220          1210      1200
MON95- CTAGATCGATATTTCCATCCAT---CATCTTAA-ACTCGTAACTATGAA---GAT---GT
         : :  :  :   : : :: :   :: :  :: :     ::  :::::   :::   ::
NC_024 GGAAAAGGGCAAACCAAACCCTATGCAACGAAACAAAAAAAATCATGAAATCGATCCCGT
  84633780  84633790  84633800  84633810  84633820  84633830
    1190      1180         1170           1160      1150       1140
MON95- ATGGCACACACATACAGATA---CAAAATT-----AATAAATCCACCAGGTAGCTTG-AA
        ::    ::   :: ::  :   ::  :::     :: : :  :::  :  :: : : ::
NC_024 CTGCGGAACGGCTAGAGCCATCCCAGGATTCCCCAAAGAGAAACACTGGCAAGTTAGCAA
  84633840  84633850  84633860  84633870  84633880  84633890
             1130      1120      1110       1100       1090
MON95- ACAGTATCCTACTCCGATCTAGAACGACCGCCCAACCA-GACCAC-ATCATCACAAACAT
        ::: :  :   :: ::  :: :     :  ::    : :: : : : :: :::  :  :
NC_024 TCAGAA--CGTGTCTGACGTACAGGTCGCATCCGTGTACGAACGCTAGCAGCACGGATCT
  84633900    84633910  84633920  84633930  84633940  84633950
     1080      1070      1060      1050      1040      1030
MON95- GAACATGAACATGTTTGCTCTAACACAAACATGAACAGAAGTAGAACTACCGGGCCCTAA
        ::::  :::: :   : ::::::::::::::::::::::::::::::::::::::::::
NC_024 -AACACAAACACG---GATCTAACACAAACATGAACAGAAGTAGAACTACCGGGCCCTAA
     84633960     84633970  84633980  84633990  84634000  84634010
     1020      1010      1000       990       980       970
MON95- CCATGGACCGGATCGCCGATCTAGAGAAGGTAGAGAGAGGGGGGGGGGGAGGATGAGCGG
       :::::::::::: :::::::::::::::::::::::: :::::::::::::: ::::::
```

```
NC_024 CCATGGACCGGAACGCCGATCTAGAGAAGGTAGAGAG-GGGGGGGGGGGGAGGACGAGCGG
        84634020  84634030  84634040   84634050  84634060  84634070
      960       950       940       930       920       910
MON95- CGTACCTTGAAGCGGAGGTGCCGACGGCTGGATTTGGGGGAGATCTGGTTGCGTGTGTGT
       :::::::::::::::::::::::::::: :::::::::::::::::::::: ::::::::
NC_024 CGTACCTTGAAGCGGAGGTGCCGACGGGTGGATTTGGGGGAGATCTGGTTGTGTGTGTGT
         84634080  84634090  84634100  84634110  84634120  84634130
      900       890       880       870       860       850
MON95- GCGCTCCGAACGAACACGAGGTTGGGGAAAGAGGGTGTGGAGGGGGTGTCTATTTATTAC
       :::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 GCGCTCCGAAC-AACACGAGGTTGGGGAAAGAGGGTGTGGAGGGGGTGTCTATTTATTAC
         84634140   84634150  84634160  84634170  84634180
      840       830       820       810       800       790
MON95- GGCGGGCGAGGAAGGGAAAGCGAAGGAGCGGTGGGAAAGGAATCCCCCGTAGCTGCCGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 GGCGGGCGAGGAAGGGAAAGCGAAGGAGCGGTGGGAAAGGAATCCCCCGTAGCTGCCGGT
84634190  84634200  84634210  84634220  84634230  84634240
      780       770       760       750       740       730
MON95- GCCGTGAGAGGAGGAAGAGGCCGCCTGCCGTGCCGCCTCACGTCTGCCGCTCCGCCACGC
       ::::::::::::::: :::::::::::::::::: ::::::::::::::::::::::::
NC_024 GCCGTGAGAGGAGGAGGAGGCCGCCTGCCGTGCCGGCTCACGTCTGCCGCTCCGCCACGC
84634250  84634260  84634270  84634280  84634290  84634300
      720       710       700       690       680       670
MON95- AATTTCTGGATGCCGACAGCGGAGCAAGTCCAACGGTGGAGCGGAACTCTCGAGAGGGGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 AATTTCTGGATGCCGACAGCGGAGCAAGTCCAACGGTGGAGCGGAACTCTCGAGAGGGGT
84634310  84634320  84634330  84634340  84634350  84634360
      660       650       640       630       620       610
MON95- CCAGAGGCAGCTACAGAGATGCCGTGCCGTCTGCTTCGCTTGGCCCGACGCGACGCTGCT
       :::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 CCAGAGGCAGCGACAGAGATGCCGTGCCGTCTGCTTCGCTTGGCCCGACGCGACGCTGCT
84634370  84634380  84634390  84634400  84634410  84634420
      600       590       580       570       560       550
MON95- GGTTCGCTGGTTGGTGTCCGTTAGACTCGTCGACGGCGTTGAACAGCCTGTCATTATCTA
       :::::::::::::::::::::::::::::::::::::::: ::::: ::: :::::::::
NC_024 GGTTCGCTGGTTGGTGTCCGTTAGACTCGTCGACGGCGTTTAACAGGCTGGCATTATCTA
84634430  84634440  84634450  84634460  84634470  84634480
      540       530       520       510        500       490
MON95- CTCGAAACAAGAAAAATGTTTGCTTAGTTTTTTT-ATTTCTTAAAGGGTATTTGTTTTAT
       :::::::::::::::::::::: :::::::::::: :::::::::::::::::::::: ::
NC_024 CTCGAAACAAGAAAAATGTTTCCTTAGTTTTTTTAATTTCTTAAAGGGTATTTGTTTAAT
84634490  84634500  84634510  84634520  84634530  84634540
       480       470       460       450       440        430
MON95- TTGTAGTCAATTTATTTTATTTCATTTTATATCTAAATTATTAAAT-AAAAAACTAAAAT
       :: :::::: :::::::::::  :::::::::::::::::::::: :::::::::::::
NC_024 TTTTAGTCACTTTATTTTATTCTATTTTATATCTAAATTATTAAATAAAAAAACTAAAAT
84634550  84634560  84634570  84634580  84634590  84634600
        420       410       400       390        380         370
MON95- AGAGTTTTAGTTTTAAAAAATTTAGAGACTAAAA-AGAATAAAATGGATGTACT--AAAA
       ::::::::::::::    ::::::::: :::::: :::::::::: ::::::::  ::::
```

```
NC_024 AGAGTTTTAGTTTT-CTTAATTTAGAGGCTAAAATAGAATAAAATAGATGTACTAAAAAA
84634610  84634620   84634630  84634640  84634650  84634660
          360       350       340       330       320       310
MON95- ATTAGTCTATAGAAACCATCAACCCTAAATCCTAAATGGATGTACTAATAAAATGGATGA
       :::::::::::: ::::::: ::::::::: ::::::::::::::::::::::::::::
NC_024 ATTAGTCTATAAAAACCATTAACCCTAAACCCTAAATGGATGTACTAATAAAATGGATGA
84634670  84634680  84634690  84634700  84634710  84634720
          300       290       280       270       260       250
MON95- AGTATTATATAGGTCAAGCTATTTGCAAAAAAAACAGAGAGATCACATGCACACTAAAAA
       :::::::::::::: :::::::::::::::::: :: :::: :::::::::::::::::
NC_024 AGTATTATATAGGTGAAGCTATTTGCAAAAAAAA-AG-GAGAACACATGCACACTAAAAA
84634730  84634740  84634750  84634760    84634770  84634780
          240             230       220       210       200
MON95- GATAAAACTGTAGA------TTGTCAAAATATTCAATTATCCTTTAGACCATGTCTAGCA
       ::::::::::::::      :::::::::::: :::::: :::::::::::::::::: :
NC_024 GATAAAACTGTAGAGTCCTGTTGTCAAAATACTCAATTGTCCTTTAGACCATGTCTAACT
   84634790  84634800  84634810  84634820  84634830  84634840
       190       180       170       160       150       140
MON95- GTTTATTTATATGATCCTCTAAAACACTAATATTATTTTAGTATTATAGACTATATTATT
       ::: :::::::::::: :::::::::::: :::::::: ::::: :::::: :::::::::
NC_024 GTTCATTTATATGATTCTCTAAAACACTGATATTATTGTAGTACTATAGATTATATTATT
   84634850  84634860  84634870  84634880  84634890  84634900
       130       120       110       100        90        80
MON95- TGTAGAGTGAAGTTTAAATATATGTATAGAGATAGATAAACTACACTTCAAATAAGTGTG
        ::::::: :::::::::::::::::::: :::::::::::::: ::::::::: :::::::
NC_024 CGTAGAGTAAAGTTTAAATATATGTATAAAGATAGATAAACTGCACTTCAAACAAGTGTG
   84634910  84634920  84634930  84634940  84634950  84634960
         70        60        50        40        30        20
MON95- AC-AAAAAAATATGTGGTAATTTTTTATACTTTAGACATGCAATGCTCTTTATCTCTAGA
       :: :::::::::::::::::::::::::  ::::::::::::::::: ::::::::::::
NC_024 ACAAAAAAAATATGTGGTAATTTTTTATAACTTAGACATGCAATGCTCATTATCTCTAGA
   84634970  84634980  84634990  84635000  84635010  84635020
         10
MON95- GAGGGGCACGAC
       ::::::::::::
NC_024 GAGGGGCACGACCGGGTCACGCTGCACTGCAGTCTACCTTGCCATGCCGCCGGATTCAGC
   84635030  84635040  84635050  84635060  84635070  84635080
>--
rev-comp initn: 1922 init1: 1113 opt: 2507  Z-score: 1167.5  bits: 235.8 E(14397): 4.5e-59
banded Smith-Waterman score: 2507; 94.9% identity (94.9% similar) in 568 nt overlap (2007-
1451:84633065-84633628)
                                        2000      1990      1980
MON95-                                GACCTGCAGAAGTATCACCAAACAACAGGGT
                                       : :::::::::: ::::::::::::::::
NC_024 TTGCCAGTCAGGGTTTTCACAAAGATCTGCATCTGCAGAAGTAACACCAAACAACAGGGT
     84633040  84633050  84633060  84633070  84633080  84633090
           1970      1960      1950      1940      1930       1920
MON95- GAGCATCGGACAAAAGAAACAGTACCAAGCAAATAAATAGCGTATGAAGGCAGGGCT-AA
       ::::::: :::::::::::::::::::::::::::::::::::::::::::::::::: ::
NC_024 GAGCATC-GACAAAAGAAACAGTACCAAGCAAATAAATAGCGTATGAAGGCAGGGCTAAA
```

```
     84633100   84633110  84633120  84633130  84633140  84633150
            1910      1900      1890      1880      1870      1860
MON95- AAAATCCACATATAGCTGCTGCATATGCCATCATCCAAGTATATCAAGATCAAAATAATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 AAAATCCACATATAGCTGCTGCATATGCCATCATCCAAGTATATCAAGATCAAAATAATT
      84633160  84633170  84633180  84633190  84633200  84633210
            1850      1840      1830      1820      1810      1800
MON95- ATAAAACATACTTGTTTATTATAATAGATAGGTACTCAAGGTTAGAGCATATGAATAGAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 ATAAAACATACTTGTTTATTATAATAGATAGGTACTCAAGGTTAGAGCATATGAATAGAT
      84633220  84633230  84633240  84633250  84633260  84633270
            1790      1780      1770      1760      1750      1740
MON95- GCCGCATATGCCATCATGTATATGCATCAGTAAAACCCACATCAACATGTATACCTATCC
       :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 GCTGCATATGCCATCATGTATATGCATCAGTAAAACCCACATCAACATGTATACCTATCC
      84633280  84633290  84633300  84633310  84633320  84633330
            1730      1720      1710      1700      1690      1680
MON95- TAGATCAATATTTCCATCCATCATCTTAAACTCGTAACTATGAAGATGTATGGCACACAC
       :::::: ::::::::::::   ::::::::::::::::::::::::::::::: :::::::
NC_024 TAGATCGATATTTCCATC---CATCTTAAACTCGTAACTATGAAGATGTATGACACACAC
      84633340  84633350     84633360  84633370  84633380  84633390
            1670      1660      1650      1640      1630      1620
MON95- ATAAAGATACAAAATTAATAAATCCACCAGGTAGTTTGAAACAGTATTCTACTCCGATCT
       ::: :: : :::::::::::::: ::::::::::::::::::::::::::::::::::::
NC_024 ATACAGTTCCAAAATTAATAAATACACCAGGTAGTTTGAAACAGTATTCTACTCCGATCT
        84633400  84633410  84633420  84633430  84633440  84633450
                1610      1600      1590      1580       1570
MON95- A--------GAACGACCGCCCAACCAGACCACATCATCACAACCAAGCGAA-AAAAAGCA
       :        ::::::::::::::::: :::::::::::::::::::::::: ::::::::
NC_024 AGAACGAATGAACGACCGCCCAACCACACCACATCATCACAACCAAGCGAACAAAAAGCA
        84633460  84633470  84633480  84633490  84633500  84633510
           1560      1550      1540      1530      1520      1510
MON95- TCTCTGTATATGCATCAGTAAAACCCGCATCAACATGTATACCTATCCTAGATCGATATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NC_024 TCTCTGTATATGCATCAGTAAAACCCGCATCAACATGTATACCTATCCTAGATCGATATT
        84633520  84633530  84633540  84633550  84633560  84633570
           1500      1490      1480       1470      1460      1450
MON95- TCCATCCATCATCTTAAACTCGTAACTATGAAGATGTA-GGCACATACATACAGATCCTT
       ::::::::::::::: :: :::::::::::::: ::::: :::::: ::::::::::::
NC_024 TCCATCCATCATCTTCAATTCGTAACTATGAATATGTATGGCACACACATACAGATCCAA
        84633580  84633590  84633600  84633610  84633620  84633630
            1440      1430      1420      1410      1400      1390
MON95- TAATAAATCCACCAGGTAGTTTGAAACAGTATTCTACTCCGATCTAGAACGACCCTTTAT

NC_024 AATTAATAAATCCACCAGGTAGTTTGAAACAGAATTCTACTCCGATCTAGAACGACCGCC
        84633640  84633650  84633660  84633670  84633680  84633690
```

The alignment between the MON 95379 *Zm.Ubq* element and the corresponding region of a conventional *Zea mays* chromosome five, which encodes the endogenous homolog, is broken into two main alignments derived from T-DNA element residues 1-1385, and 1451-2007 (Figure L-1). Each of these regions of alignment required numerous gaps and mismatches to align and displayed 86.1% and 94.9% identity respectively (Figure L-1). This level of alignment fragmentation along with the internal gaps and mismatches within the alignments, is consistent with the non-uniform coverage and over representation of unpaired reads observed in the conventional control dataset (Figure L-2). This is the result of some quantity of endogenously sourced reads having sufficient similarity to map to portions of the T-DNA *Zm.Ubq* element, while their mate pairs are of insufficient identity for placement, resulting in an over-representation of unpaired reads (Figure L-2, panel 1).

This is fragmentation and dissimilarity further substantiated in the conventional control mapping, where it can be observed that reads are unable to align with uniform coverage across the entirety of the *Zm.Ubq* element (Figure L-2, panel 3). Another indication that these reads are sourced form an endogenous element and not a secondary insert is that the mapped reads are constrained specifically to the *Zm.Ubq* element, which would be highly unlikely in the event of a secondary insert fragment (Figure L-2).

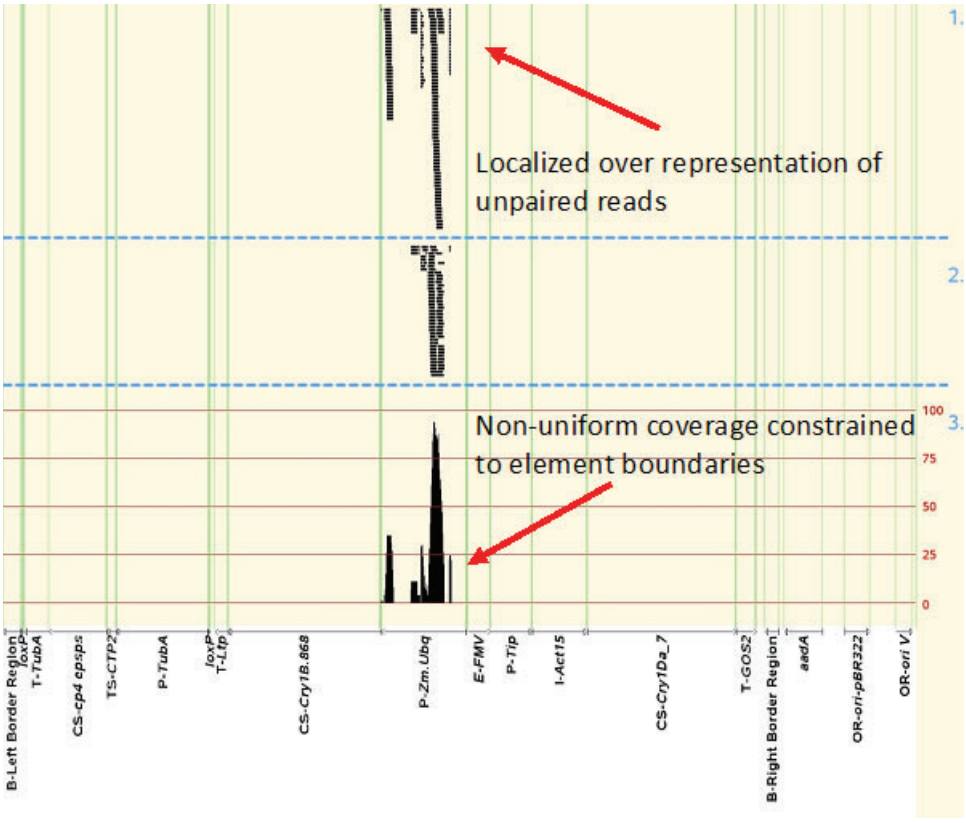


Figure L-2. (Derived from Figure IV-5) Read Mapping of Conventional Maize LH244 Versus PVZMIR522223 with Supplemental Annotations

This LH244 conventional control mapping demonstrates the expected level and areas of mapping that might be expected due to the presence of a maize native element (*Zm.Ubq*) within the T-DNA. Notably, all reads are constrained to the known common element boundaries, and coverage is non-uniform across the element indicating some level of sequence deviation relative to that of the T-DNA element.

When comparing the mapped reads from the conventional control sequencing back to that of the MON 95379 sample F4 T-DNA mapping it becomes clear that the additional unpaired reads captured across the *Zm.Ubq* element result in virtually superimposable signatures (Figure L-3).

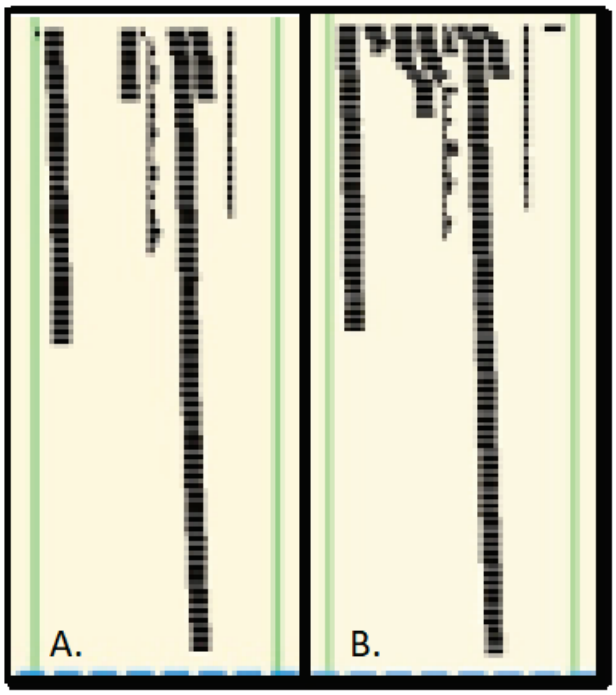


Figure L-3. Isolated Un-Paired Read Regions Corresponding to the *Zm.Ubq* Elements in MON 95379 Sample F4 and the LH244 Conventional Control.

Panel A corresponds to MON 95379 sample F4 (Figure IV-6), and Panel B to LH244 control mapping (Figure IV-5). Notably the control and samples demonstrate qualitatively identical signatures indicating that all commonality across these regions is derived from the endogenous LH244 *Zm.Ubq* homolog.

This identical presence and relative quantity of unpaired reads along with the isolated regions of increased depth seen in the characterized MON 95379 generation (Figure L-4) shows that all additional sequencing derived from this location is both expected due to the presence of the endogenous *Zm.Ubq* homolog in conventional maize, and not due to a secondary insertion. In contrast, if a secondary copy of a portion of the T-DNA were present, it would result in depth of sequencing being doubly present across the entire secondary insert/insert fragment, with a tripling of coverage in the areas sourced from the endogenous copy.

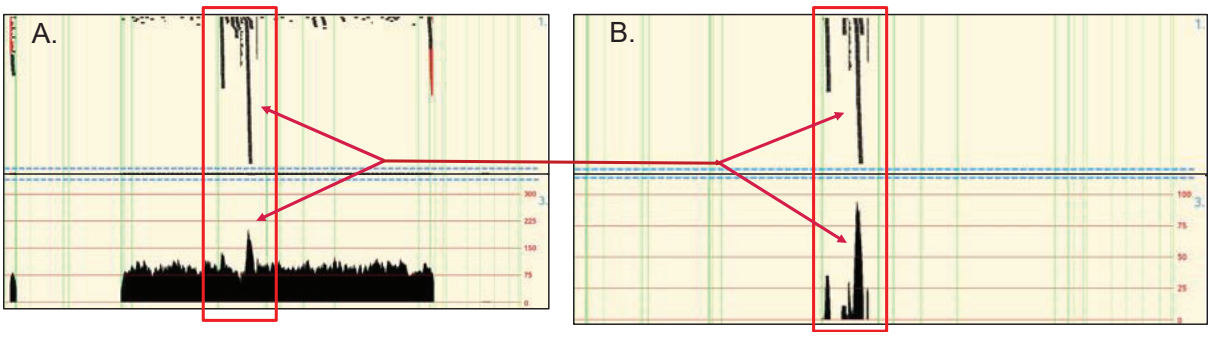


Figure L-4. Side by Side Annotated comparison of Unpaired Read and Depth Plots of the MON 95379 Insertion and the LH244 Conventional Control.

Section A displays panels 1 and 3 from Figure IV-6. Section B displays panels 1 and 3 from Figure IV-5. Red boxed areas represent location of *Zm.Ubq element*. Arrows pointing to areas of greatest depth observed and corresponding peak observable unpaired and paired (not pictured) reads in the LH244 control dataset.

Although some DNA from the LH244 control was identified, its presence was expected due to the shared element *Zm.Ubq*. A review of this identified region between the control and test samples demonstrates highly congruent levels of capture and is therefore accounted for in the LH244 conventional control dataset. Overall, these mapping signatures and lack of identified secondary junction sites supports a single T-DNA insertion in MON 95379.

L.5. Additional Information Regarding Flank Capture and Coverage in the Characterization of the DNA Inserts in MON 95379 (Section IV.B)

The NGS mapping portion of the experiment is designed to identify any and all plasmid insertions within a genome, and simultaneously absence of vector backbone, by mapping to the entirety of the transformation construct. The inclusion of the flanking genomic regions in the analysis is not feasible because it 1) pre-supposes knowledge of these insertion sites and their respective junctions and would confound our ability to detect potential secondary or higher order inserts, and 2) would impact the plasmid "backbone" region of the figure, confounding the ability to conclusively determine the absence of any backbone sequence. However, the coverage over the flanking genomic regions as captured by the NGS experiments are analyzed over five breeding generations as part of a generational stability analysis, and the alignment of the consensus sequences of the flank-junction spanning reads are illustrated in Appendix K; Figure K-19. Notably, the flank depth is at minimum the same depth of all truncated reads terminating at the junction point. The Y-Axis in panel 3 of the mapping represents the raw depth of reads across the plasmid sequence. Appendix K also includes 1) mapping figures for the subsequent generations (F4F1, F5, F5F1, and F6F1), controls, and reference substances, and 2) tables summarizing coverage statistics for all samples including controls (Tables K-1 and K-2).

L.6. Additional Information Regarding Sequencing Utilized in the Mapping Process for the Conformation of the Absence of Cre Recombinase (Section IV.B.4)

The reads used to confirm the absence of the Cre recombinase cassette are the same reads generated and used to confirm the presence of the single copy of the T-DNA (generation F4). Likewise, the process and parameters used are identical to those used for all samples as described in Section B.9. The difference between the processes to show the absence of the Cre recombinase cassette and the characterization of the inserted T-DNA is the transformation vector used as a reference sequence during the mapping process. Read mappings to the transformation vector containing the Cre recombinase machinery (PV-ZMOO513642) are provided in Figure IV-7, and total sequencing generated for F4 is provided in Appendix K (Table K-1). Notably the total sequencing generated for this sample, which was then mapped to both of these transformation vectors, was 191.54 gigabases (Gb).

L.6.1. Clarification of Whole Genome Sequence Analysis (Appendix B)

Although whole genome sequencing was done on multiple generations, these data were generated to be used in a read mapping experiment. As such no de-novo assembly was conducted on this data, and therefore no assembly software was used.

L.6.2. Clarification on B.13 Rejected Data

Data was rejected in three instances during the molecular characterization of MON 95379: 1) During the preparation of PCR amplicons for the insertion site analysis. 2) During the trimming and assembly of insert and insertion site sequences. 3) During the whole genome sequencing of MON 95379. The context and impact of these rejections are explained in greater detail below:

1. In order to determine the insertion site sequence a PCR is conducted on LH244 DNA (conventional control). In one instance the amplification was not sufficient to support the downstream purification and sequencing of the amplicon causing the samples were rejected, and the PCR to be repeated.

2. The analysis of the insert and insert sequence is conducted using CLC workbench (a commercially available software). During the analysis, some sequences were automatically rejected by the CLC software at two different stages of the analysis process, read trimming and read mapping. The reads that failed to meet default quality settings were automatically rejected during the trimming process. During the mapping process, additional reads were automatically rejected by the CLC workbench software if they failed to map to the reference sequence, if the sequence files contained no data, or if the sequence data was of insufficient quality.

3. A limited number of whole genome sequencing runs were rejected for MON 95379 generation F4F1 and F6F1 because of either the presence of PV-ZMIR522223

contamination or insufficient coverage. Additional sequencing runs were generated from the same starting materials to reach the targeted read depths.

In all instances, the rejected data were assessed to have no effect on the quality, integrity, or conclusions of the study.

L.7. Additional Information Regarding Binding Domains Located in the Sequences of Cry1B.868 and Cry1Da_7 (Appendix C.1.3 and D.1.3)

Mass fingerprint analysis is a standard technique used to confirm the identity of a protein in a matrix. The design and intent of the mass fingerprint analyses provided to support the characterization of Cry1B.868 and Cry1Da_7 as expressed in MON 95379 was not to obtain full amino acid sequence coverage of the proteins. Rather, the purpose of this analysis was to confirm the identity of each protein and thereby validate the subsequent equivalency assessment. The identity of the MON 95379- and *Bt*-produced Cry1B.868 and Cry1Da_7 proteins was confirmed by mass fingerprint analysis, where very high sequence coverage was observed for all the proteins tested.

The Cry1B.868 and Cry1Da_7 proteins are classical 3-domain Cry proteins. As such, the receptor binding sites are located in domains II and III (BenFarhat-Touzri et al., 2019; Bravo et al., 2013; Pigott and Ellar, 2007; Wang et al., 2019). Domains II and III fell between amino acid position 242 to 606 for Cry1B.868 and 225 to 567 for Cry1Da_7. Despite the technical limitation of the mass spectrometry technology, there were significant coverages within those amino acid ranges for both Cry1B.868 and Cry1Da_7 proteins. Based upon the peptide maps in Figures C-2, 96% of the amino acid sequence in the region that contains the receptor binding domain was confirmed for both MON 95379- and *Bt*-produced Cry1B.868. Based upon the peptide maps in Figures D-2, 100% of the amino acid sequence in the region that contains the receptor binding domain was confirmed for both MON 95379- and *Bt*-produced Cry1Da_7. The high percentage coverage of the mass fingerprint analyses provide clear support for the sequence similarity between the MON 95379- and *Bt*-produced proteins. In addition, the DNA sequences of genes of the expressed MON 95379 (Section IV.C) and DNA obtained from the *Bt* cell paste from which the proteins were purified (mentioned in Sections C.2.1 and D.2.1) had 100% coverage and the sequences aligned perfectly. It is evident from these data that the receptor binding domains between the MON 95379- and *Bt*-produced proteins are equivalent.

L.8. Additional Clarification Regarding the Least-Squares Means and Standard Errors (SE) Presented in Table VI-1 of the Compositional Assessment of MON 95379

The least-squares means and standard errors (SE) presented in Table VI-1 are model-based estimates obtained from the linear mixed model analysis of composition data. The SE values are calculated using the model-based estimates of the random effects and residual variance, as well as the sample sizes of the treatments (Milliken and Johnson, 2009). Under the model assumption of homogeneity of variance, all treatments are assumed to have the same residual variance.

Additionally, the sample size of each treatment is equal, i.e. four replicates per site and treatment. Therefore, the SE values are identical for all treatments.

L.9. Additional Information Regarding Insect Activity Spectrum Testing for Cry1B.868 and Cry1Da_7 (Section VII)

Activity spectrum screening is not intended to provide a comprehensive assessment of specificity or cover all ecological functions or guilds, but rather provides a reasonable early indication of activity at levels expressed in the plant that contributes to problem formulation and informs NTO safety testing (Bachman et al., 2017; Bachman et al., 2013; Romeis et al., 2008). When characterizing activity of these proteins, the activity spectrum data are not interpreted in a vacuum. Information gathered for problem formulation provides a strong history of safe use, including limited insecticidal activity spectra for Cry1 proteins (Romeis et al., 2006) . Multiple species from the target order are typically selected to understand the variability in potency within the target order. Additional species outside of the target order are typically selected for activity spectrum screening and taxa selection is based on multiple criteria including representation of other key orders, assay availability, historical precedence, and relatedness to the target order. For MON 95379 maize, selected taxa were representative of the breadth of testing for previous familiar *Bt* proteins (e.g., MON 89034 maize). Four lepidopteran, four coleopteran species, and two hemipteran species were selected based on such criteria and, as such, activity spectrum testing is not intended to serve as a comprehensive safety assessment accounting for all feeding guilds and functions (Section VII.A). In the case of Cry1B.868, there was information from the literature that Cry1B may have activity against chrysomelids and therefore, focus on chrysomelids provides especially useful information. Also, chrysomelids have often been susceptible to coleopteran-active *Bt* proteins. Additionally, information from the subsequent tier I NTO studies also feeds back into an evergreen problem formulation further contributing to our understanding of "activity spectrum" in a broad sense. Conclusions of risk for Coleoptera are ultimately driven by margins of exposure established with the coccinellid and carabid NTO studies.

Interactions are unlikely to result in effects to species when the individual components are present below their no-effect level (Levine and Borgert, 2018). Results of the interaction study supports the conclusion that Cry1B.868 and Cry1Da_7 act independently and thus can be assessed independently (Appendix J). This supports the conclusion that the combination of Cry1B.868 and Cry1Da_7 is unlikely to exhibit a different spectrum activity from those of the individual proteins.

The reference to activity spectrum screening results, in the context of assessing results from adult lacewing testing, was part of a weight-of-evidence approach to reiterate what was known about potential activity of Cry1B.868 based on information developed during problem formulation (Section VII.C.1). As stated above, the primary purpose of activity spectrum is not to provide a comprehensive assessment on specificity and safety, but rather to inform the species selection in the NTO studies that drive the safety assessment. The interpretation of activity spectrum results with respect to Neuroptera is not based on phylogenetic distance, but rather that in initial screening, effects were only observed in Lepidoptera, consistent with the history of safe use developed with familiar Cry1-family proteins and their typical spectra of insecticidal activity (e.g., Lepidoptera).

For clarity, activity spectrum was not used to make conclusions of risk to Neuroptera. The result observed in *C. carnea* was addressed through additional testing, including a neuropteran species where a more sensitive larval life-stage could be evaluated. Conclusions of risk were based on minimal exposure from cultivation of MON 95379 maize in combination with results from the additional testing in green lacewing larvae and adults.

In conclusion, activity spectrum results are used as part of a weight-of-evidence approach along with mode-of-action, exposure assessments, and laboratory NTO testing to assess potential impacts on organisms beneficial to agriculture. Ultimately, risk conclusions are driven by the margins of exposure established during laboratory NTO testing of species representing key ecological functions with relevant exposure to MON 95379 maize.

L.10. Additional Information Regarding Potential Exposure to Cry1B.868 and Cry1Da_7 Through Prey (Section VII.B.2)

With respect to the estimates on potential exposure to beneficial predators through herbivore prey (Section VII.B.2), the maximum levels of Cry1Ac observed in spider mites by Obrist and colleagues correspond to approximately 1.5-fold above that expressed in plant tissue. A broader survey of the literature indicates that relative concentrations in mites can vary significantly between different Cry proteins and experiments and can typically range from 0.1× to 1.5× of that expressed in plants (Kim et al., 2020; Obrist et al., 2006b; Svobodavá et al., 2017). Spider mites are typically considered a worst-case scenario with respect to potential exposure to beneficial predators through prey. However, Cry protein concentrations in prey species such as aphids and juvenile lepidoptera are much lower than those found in spider mites. Given the generalist nature of predation by beneficial predators in the agricultural ecosystem, the magnitude of exposure to Cry proteins would be expected to be less than the worst-case scenario: a diet 100% consisting of spider mites feeding on MON 95379 maize. This would be consistent with previous published risk assessments that advocate for a value of 0.2× plant expression to reflect a realistic exposure scenario for generalist predators in the agricultural ecosystem (Raybould and Vlachos, 2011). Therefore, based on the potential variability in experiments, prey diversity, uptake of different Cry proteins, plant expression values were considered to be a reasonably conservative estimate of exposure through prey. However, it should be noted that focusing the assessment on an absolute worst-case scenario would not change the conclusions of the risk assessment for beneficial arthropods exposed via this route. Applying a 1.5-fold exposure factor to EECs for beneficial predators results in MOEs >20 for Cry1B.868 and >12 for Cry1Da_7 (with the exception of *C. maculata* where the MOE is >4 due to a correction to the test concentration, but no adverse effects on survival were observed). Therefore, the margins of exposure determined from Tier I NTO testing are sufficient to account for a worst-case exposure scenario of 1.5-fold above plant expression.

L.11. Additional Information Regarding Environmental Fate of Cry1B.868 and Cry1Da_7 in Soil (Section VII.B.2)

The calculations for the predicted environmental concentrations (PECs) for Cry1Da_7 and CryB.868 are provided at the beginning of the Soil Invertebrates exposure section (Section VII.B.2). The PEC values serve as a starting point in the exposure assessment for soil organisms in providing a conservative estimate of soil concentrations assuming the entire biomass of a field is incorporated into the top 6 in (15 cm) of soil The PEC values do not take into account other factors that may influence soil concentrations such as degradation. The results of the soil degradation study were used to refine potential exposure to soil organisms. For Cry1B.868, which exhibited rapid dissipation in soil, the conclusion was made that this protein was unlikely to persist or accumulate and thus the EEC for Cry1B.868 is the same as the PEC (0.35 µg/g; compare Table VII-2 and Table VII-5; please note that the EEC was rounded to one significant digit). Results from the laboratory study indicated Cry1Da_7 did show potential for persistence in sandy loam soil. Therefore, the accumulation model described in Section VIII.B.2 was used to account for potential accumulation of Cry1Da_7 under very conservative assumptions for both half-life duration (i.e., up to 1 year) and 25 consecutive years of planting MON 95379 without rotation. The 2-fold increase in soil concentration predicted by the model was then applied to the PEC to generate the EEC for Cry1Da_7. While soil incorporation was deemed the most ecologically relevant route of exposure to evaluate risk to soil organisms, test concentrations were deliberately selected to be sufficiently conservative to account for more direct exposure scenarios if necessary.

Several factors influence the potential degradation rate of proteins in the environment including temperature, rain events, sunlight, and interaction with biotic factors such as robustness of the microbial community. The soil degradation study is based on general principles outlined in the EPA test guideline OPPTS 835.4100. The purpose of this study is to characterize the likelihood of persistence in soils given the characteristics of the proteins and soil properties while minimizing variability in the environment. It is reasonable to expect fluctuation in temperatures could impact enzymatic processes involved in degradation, but as noted above, it is only one of several factors. Environmental and soil temperatures are also cyclical in nature and even if periods of cold or frozen soil could slow degradation, inevitable seasonal warming would eventually lead to conditions amenable for degradation. This conclusion would be consistent with results from field monitoring studies where *Bt* proteins would be exposed to a full array of environmental factors and these studies have shown that *Bt* proteins do not persist or accumulate in the soil environment (Ahmad et al., 2005; Dubelman et al., 2005; Gruber et al., 2012; Head et al., 2002; Shan et al., 2008). Persistence in soil and dissipation rates are determined by a number of factors. Clay soils, often associated with higher cation-exchange capacities, can generally bind macromolecules such as Cry proteins and nucleic acids more tightly than other soil types like sand and silt (Gruber et al., 2012; Icoz and Stotzky, 2008). Binding to clay particles can render proteins less available to enzymatic degradation and result in prolonged dissipation. However, other factors can also influence dissipation rates including protein characteristics, organic matter content, robustness of the microbial communities, soil moisture, and pH. Given the variety of environmental factors that influence degradation, it is not unexpected that relative degradation rates across soil types would

vary across studies and proteins. Generally, microbial activity is thought to be a principle factor in degradation rates of free protein (Icoz and Stotzky, 2008). Radiolabeled ^{14}C-Cry1Ab applied to soils resulted in rapid CO_2 evolution and the ^{14}C-label was incorporated into the microbial biomass indicating that the applied Cry1Ab protein was being utilized as a microbial nutrition source (Valldor et al., 2015). This result is consistent with the notion that proteins are typically amendable to biodegradation in the environment. Guidance from EPA test guideline OPPTS 835.4100 recommends that soil dissipation tests generally not be conducted beyond 120 days due to the potential decline of microbial communities in test systems that are isolated from the environment and natural replenishment. For this reason, laboratory studies represent a more conservative approach to assess the potential for persistence of Cry proteins in soils.

The likelihood that test conditions resulted in an underestimate of exposure in soil is low. Cry1B.868 dissipated rapidly in soil with DT_{50} values comparable to other proteins that have been shown to not persistent in the environment. Cry1Da_7 also had DT_{50} values in two soil types that were comparable to previously commercialized *Bt* proteins (Icoz and Stotzky, 2008). For sandy loam soil, a first-order accumulation model based on highly conservative assumptions was used to generate a worst-case exposure levels to soil organisms in this soil type. These assumptions included a conservative estimated DT_{50} of 1 year, well beyond the duration of the study, and does not account for crop rotation. This calculation does not consider other environmental factors that would be expected to influence degradation rates. The result of the model was used to apply an exposure factor to the Cry1Da_7 PEC to generate a conservative EEC for soil organisms to characterize risk. Therefore, the assumptions applied provide a reasonable worst-case estimate and it is unlikely the results from the laboratory soil degradation study would lead to an underestimate of soil concentrations. Furthermore, the margins of exposure for Cry1Da_7 generated in Tier I testing for soil organisms was >2500, after calculating highly conservative exposure estimates, and is sufficiently protective of soil organisms.

A similar dissipation profile was observed for Cry1Ac (expressed in MON 87701 soybean), which was tested in a soil degradation study to evaluate potential persistence in soil as part of the registration with EPA (U.S. EPA, 2010b). At the end of the 181-day incubation period, 72% to 96% of the Cry1Ac had degraded regardless of soil texture, pH, clay content, or the method used to analyze the soil. The DT_{50} of Cry1Ac in soils was ranged from 0.4 – 13 days. However, modeling of the dissipation curves indicated that the DT_{90} was >181d for all three soil types, including a sandy soil. Despite the inability to calculate DT_{90} times in the laboratory study, field studies indicate that the Cry1Ac protein does not persist in sandy loam and silt loam soils under field conditions following multiple consecutive years of planting Bollgard cotton (Head et al., 2002). Results from Cry1Ac in soybean and cotton indicate that while Cry1 proteins can exhibit relatively longer dissipation times, including in sandy soils under laboratory conditions, it is unlikely to result in persistence in the field. This further supports the conclusion that concentrations of Cry1Da_7 protein in sandy loam soil are expected to be low (≤0.08 µg/g) and Cry1Da_7 is unlikely persist or accumulate in the environment.

Environmental factors that impact potential degradation are taken into account in open field trials and published field surveys have demonstrated that Cry proteins do not persist or accumulate in

the environment (Ahmad et al., 2005; Dubelman et al., 2005; Gruber et al., 2012; Head et al., 2002; Shan et al., 2008). In a comprehensive multi-year field survey, Gruber and colleagues evaluated soils for the presence of Cry1Ab in fields that were planted with MON 810 maize for nine consecutive years (Gruber et al., 2012). Over the course of the 9-year trial, Cry1Ab was detected in soil above detectable levels at a single site 6 weeks after the 8th consecutive growing season. Notably, no Cry1Ab protein was detected at any site in any of the spring samplings prior to planting indicating that seasonal decreases in soil temperature did not result in persistence or accumulation of Cry1Ab in soil.

In review of the results with Cry1B.868 and Cry1Da_7, Cry1B.868 dissipated rapidly in all soil types and Cry1Da_7 dissipated in clay and silt soils with kinetics comparable to other *Bt* proteins (reviewed in (Icoz and Stotzky, 2008)). While a DT_{50} could not be calculated for Cry1Da_7 in sandy soil, it should be noted that no biological activity was measured in sandy loam soil at the end of the study using a sensitive bioassay with FAW. Furthermore, the study was conducted at a field incorporation rate of 10-fold above a 1× maximum field load scenario. An exposure factor derived from a conservative accumulation model was applied to account for the longer dissipation profile for Cry1Da_7 in sandy soil and margins of exposure were still >2500 indicating minimal risk of Cry1Da_7 to soil organisms, regardless of soil type.

L.12. Further Discussion of the Cry1Da_7 Results from the Soil Dissipation Study (Section VII.B.2)

ELISA analysis completed on samples generated to establish dissipation rates for Cry1B.868 and Cry1Da_7 in MON 95379 in the soil dissipation study was conducted under GLP using a fully validated method with documented protocols. All assays contain positive and negative controls and assays have accept and reject type criteria as quality control measures.

Assay to assay variation likely played a role in sample variability observed when comparing sample concentrations on Day 121 to Day 213. Samples were run on two separate assays on separate days. The first set contained all samples up to and including Day 121. The second set of samples contained the samples collected at Day 213 only. It was deemed necessary to generate the Day 213 sample to more fully characterize the dissipation rates for Cry1Da_7.

Sample to sample variability is low as shown in the table below. Reps were from independent vessels. Calculated standard error values for the MON 95379 soil dissipation study are low and represent acceptable variation found when subsampling from multiple vessels.

Table L-1. Cry1Da_7 concentrations in three soil types as measured by ELISA

Cry1Da_7 incubation time (days)	Sandy Loam		Silt Loam		Clay Loam	
	sand	mean µg/g (SD)	silt	mean µg/g (SD)	clay	mean µg/g (SD)
90	1.08	0.90	0.48	0.37	0.31	0.28
90	0.88	(0.17)	0.41	(0.14)	0.35	(0.09)
90	0.74		0.22		0.18	
120	0.69	0.61	0.43	0.32	0.16	0.24
120	0.53	(0.08)	0.28	(0.09)	0.32	(0.11)
120	0.61		0.25		<LOQ[1]	
213	1.04	0.88	0.24	0.40	0.25	0.23
213	0.83	(0.14)	0.41	(0.15)	0.22	(0.02)
213	0.78		0.54		0.21	

LOQ for Cry1Da_7 is 0.063 µg protein /g sandy loam and silt loam soil, and 0.125 µg protein /g clay loam soil

L.13. Additional Information on Potential Exposure of Cry1B.868 and Cry1Da_7 to Collembola (Section VII.B.2)

Soil incorporation of post-harvest maize tissue represents a standard approach for early tier assessment of risk to soil organisms (Raybould and Vlachos, 2011; U.S. EPA, 2008; 2018b). This approach considers exposure from tilling the field into the top layers or soil and also accounts for any leaching up free protein from the degrading biomass on the soil surface into the field. The diet of Collembola is principally composed of soil hummus, mycorrhizae, and other fungal material and higher plant litter represents a relatively small proportion of the total diet (Ponge, 2000). Based on the results of laboratory testing, direct exposure of Collembola to maize litter is sufficiently accounted for in the risk assessment (Sections VII.C.1 and VII.C.2). The 95th percentile expression in forage provides a highly conservative estimate of direct exposure to terrestrial detritovores. Based on the results of the Collembola test, the margins of exposure to surface detritivores would be >71 for Cry1B.868 ((>3500 µg/g)/(48.8 µg/g)) and >45 for Cry1Da_7 ((>500 µg/g)/(11.1 µg/g)). These risk estimates are likely highly conservative as they do not take into account any

degradation of the *Bt* proteins within senescing tissue and make an unrealistic assumption that the diet of Collembola consists of 100% MON 95379 forage tissue.

L.14. Additional Information Regarding Reported Endpoints from Tier I Lacewing Tests with Cry1B.868 (Section VII.C.1)

Section VII.C.1 describes the result of the Tier I test with *Chrysoperla rufilabris* and Cry1B.868 where highest concentration tested was 700 µg/g diet. In initial testing with *Chrysoperla carnea* adults, 3500 µg/g was evaluated and a statistically significant difference in mortality was observed. Due to multiple reasons outlined in Section VII.C.1, including to the availability of a larval toxicity test, the relevance of *C. rufilabris* to North American agro-ecosystems, and the difficulties in acquiring sufficient *C. carnea* adults to run the test, additional studies were conducted with *C. rufilabris*. When no effects were observed in the larval test, an additional confirmatory test with *C. rufilabris* adults was conducted. The only relevant route of exposure for adult green lacewings to Cry1B.868 is through direct consumption of pollen so test concentrations with *C. rufilabris* were chosen to more closely reflect the magnitude of exposure from pollen consumption. Margins of exposure based on the 700 µg/g endpoint were >11 and thus sufficiently protective of green lacewing adults.

L.15. Additional Information Regarding Assay Validity Criteria for the Larval Lacewing Studies (Appendix J)

Assay validity for the green lacewing larval study is determined, in part, by mortality observed at the conclusion of the larval stage, prior to pupation (Appendix J). This assessment is distinct from the mortality observed at the end of the study reflected by adult emergence and is one of the apical endpoints evaluated in the study. This approach is consistent with established criteria outlined in OECD 239 Test Guidance for the chronic larval honey test, which requires assessment of survival at the end of the larval stage (Day 8 or 9), prior to pupation. This larval survival assessment is what's provided in the 1st paragraph of the assay validity section (Appendix J.8 and J.21). Pre-imaginal survival reported in the results section is assessed at the end of the assay when adult emergence is evaluated. Mortality that occurs in the pupal and adult emergence stages result in anddifferences between larval and pre-imaginal survival values.

L.16. Additional Information Regarding Sublethal Endpoints from Earthworm Studies with Cry1B.868 and Cry1Da_7 (Appendix J)

As described in Appendix J, no food is provided in the acute earthworm study and typically a weight loss is observed over the 14-day duration of the study. Individuals in the water control groups from the Cry1B.868 and Cry1Da_7 studies exhibited mean weight losses of 7.9% and 5.6%, respectively. For earthworms exposed to Cry1B.868, a 43% increase was observed at the 3500 µg/g treatment level and a 4% increase was observed at 700 µg/g. For Cry1Da_7, a 1% increase was observed at the 500 µg/g treatment level and a 5% decrease was observed at the 50 µg/g treatment level. The relationship between test concentrations and changes in body weight

observed in both studies indicate that the earthworms are using the Cry proteins as a nutrient source in the study. Specifically, higher test concentrations corresponded to increases in body weight. These results are consistent with the observations that earthworms can consume *Bt* proteins in soil from incorporated maize tissue and root exudates (reviewed in (Icoz and Stotzky, 2008)). While the 43% weight gain in worms exposed to Cry1B.868 at 3500 µg/g was not interpreted to be adverse as this treatment level far exceeds levels expected to be encountered in the environment, the <5% increase in body weight at 700 µg/g indicates that Cry1B.868 as expressed in MON 95379 is not expected to result in adverse effects to earthworms.

L.17. Additional Information Regarding Environmental Interactions and the Criteria for 'No Overlap' Between Stressor Ratings (Appendix H)

An environmental interaction assessment is a part of a plant characterization study that is used to assess plant interactions with the receiving environment under typical agronomic management conditions. The environmental interaction assessment is not conducted to assess for specific pests or pathogens due to a lack of a specific risk hypothesis indicating that the traits would interact with non-target herbivorous insects or pathogens in an unexpected way. However, it is used to add weight of evidence to an assessment of plant pest potential and the already long history of safe use of GE crops that have demonstrated that GE plants interact with the receiving environment in a similar manner as conventional plants with the exception of the intended effects of the introduced traits. Specific risk hypotheses related to insect-resistance traits are most thoroughly addressed by the non-target organism (NTO) data conducted according to the tiered approach (U.S. EPA, 2007). The environmental interaction assessment demonstrate that the plants were closely monitored throughout the growing season and the data are primarily used to assess for potential gross differences between the GE plant and the conventional control. Overall, this methodology provides a structure and general process by which experienced agricultural researchers evaluate plants for biotic and abiotic stressors across the growing season.

For individual site assessments, qualitative comparisons for environmental interactions assessments were made only between MON 95379 and the conventional control, and the two were considered different if their within-site ranges of raw values did not overlap. Raw data were summarized only within individual observations and sites due to the variation in temporal activity and geographical distribution of the stressors. The ranges for the damage ratings of each stressor were determined from the minimum and maximum raw damage rating values across four replications for each treatment within a site. These ranges are compared to the reference range obtained from commercial reference entries in the study to determine the relevance of any differences between the test and control. Detecting meaningful differences is a multi-layered approach. For example, the strength of the assessment lies in the comprehensiveness of approach which is obtained by collecting qualitative data 4 times across the growing season and across multiple sites that allows for robust comparative assessment of plant response to abiotic and biotic stressors. As such, any observed difference between the test and control would need to be examined in the context of data collected at other timepoints and from other sites. Also, a reference range data provides additional context to the comparative assessment. Data from the reference ranges within and across sites would further clarify the potential relevance of an observed

difference regarding whether it was a meaningful difference. Other information supporting an assessment of potential differences include in-season communication with field researchers and in-season real time QC of data. Stressors ratings showing any difference would trigger communication between the field researcher and the BCS Study Director to enable both impact on the trial at that study site as well as to evaluate the apparent differences between test and control leveraging all potential context as discussed above. Ultimately, an observed differencewould result in a site visit by the Study Director or delegate to further investigate. Therefore, even if there is no overlap between ratings, these multiple layers of comparative assessment inherent in the methodology would ensure that meaningful differences would be detected.

Overall, the strength of environmental interactions assessment is not in statistical robustness, which is not the intent of the method due to lack of plausible risk hypotheses against specific pests or pathogens. Instead, strength of the assessment is related to the comprehensiveness of the assessment that facilitates close observation of the plants across the season and across sites with flexibility for experienced agricultural researchers to assess for any potential stressor that may be causing damage at a given time, the context provided by the reference range, and close connection of the Study Director to the field researchers and data during the season. The approach as submitted is aligned with the intended qualitative nature of the assessment, provides the desired comparative assessment of the appropriate level of resolution, and avoids over-interpretation or over-analysis of the data. Also, this is published method (Horak et al., 2015) and has been consistently used by BCS in the past submissions.

L.18. Additional Information Regarding Plot Size and Measured Stressors for the Environmental Interaction Assessment (Appendix H)

The study is conducted across 8 sites that are representative of large-scale agriculture with diverse geographical and environmental conditions. Plot size was 37-39 m^2 with an overall trial area of approximately 0.7 acres. The qualitative environmental interaction data collected from the given plot size are sufficient for a fit-for-purpose assessment of environmental interactions of the GE crop intended to identify gross differences. For example, a relatively small plot size is appropriate since stressor ratings are done on plant damage symptoms as opposed to arthropod collections, which would typically require larger plot size to account for insect mobility. Relatively small plots are effectively used to differentiate between treatments in efficacy trials using damage symptoms under natural infestation conditions (Reisig et al., 2015; Souza et al., 2016). Additionally, the plot size used is part of a consistent published approach (Horak et al., 2015) and used historically in BCS submissions.

The stressors for any given site and timepoint combination were not predetermined. Measured stressors are based on the crop, growth stage, and geographical location. Prior to each data collection, the border rows in proximity of the study area were surveyed to select stressors that were actively causing damage to the crop for the subsequent evaluation in the study plots. A comprehensive list of potential stressors based on the stressors that are relevant to the crop, growth stage and geographical location of the trial was provided to experienced field researchers for data collection. Researchers selected stressors that were either actively causing damage at the time, or

in the absence of that, they were selected based on what would typically be occurring at that location and time. In addition, researchers had the flexibility to define and rate a relevant stressor not included in the above-mentioned list. The benefit of collecting data in the absence of damage provides positive confirmation that the assessment was done and that researchers were closely monitoring the plots. Recognizing that damage symptoms for certain stressors can be similar and sometimes difficult to identify to specific taxonomic levels, researchers identified stressors to the best of their ability based on knowledge of what would most likely be occurring at that site and at that time of the season. Therefore, expert local knowledge and experience with the crop was the basis for selecting appropriate stressors and estimating the range of responses that would be considered typical for maize.

L.19. Additional Information Regarding Agrochemical Use and Relative Timing of Field Observations of Arthropod and Disease Stressors for Environmental Interaction Assessment (Appendix H)

Pest management was based on pest pressure threshold to maintain trial quality and yield, mimicking commercial crop production practices (Section H.6). Based on the study protocol, field researchers apply commercially-registered agrichemicals at labeled rates for control of arthropods, diseases and weeds when they reach levels that can cause economic injury to the crop, to produce an agronomically acceptable crop. In addition to expert local knowledge and experience of field researchers with conventionally bred maize used as the basis for selecting appropriate pest management measures, the field researchers also consult with BCS regarding the type and timing of pest management. Pest management operations were performed uniformly across all plots within each site.

Pest control measures applied and timing of field observations of arthropod and disease stressors across sites are given in Tables L-2 and L-3, respectively. In summary, five out of eight sites did not receive any insecticide application. Two sites received one in-season insecticide application and one site received two insecticide applications one of which was a pre-emergent insecticide application only. Six out of eight sites did not receive any fungicide application. Only two sites received one in-season fungicide application. Therefore, relatively few insecticide and fungicide applications were used across sites so any potential effect on stressor ratings was minimal.

When pest management is applied, field researchers are expected to collect field observations on biotic and abiotic stressors either prior to the pesticide application or approximately 10 days after pesticide application to minimize impact of pesticides on environmental interaction assessment. Most of the field observations were collected 10 days after pesticide application except at site NEYO, where an environmental interaction assessment was done six days after insecticide/fungicide application due to oversight and was captured as a protocol deviation. The assessment of arthropod and disease damage “near to application time” is still useful for a comparative assessment because all plots received uniform applications of the pesticide to avoid potential confounding effects from these pest management practices. Furthermore, this visual assessment is not a measure of abundance or presence of actual arthropods that would likely be impacted following insecticide application. Additionally, any insecticide/fungicide application

was often triggered by damage symptoms by insects/diseases, thus allowing comparative assessment of arthropod/disease damage between the test MON 95379 and the conventional control. Therefore, comparative environmental interaction assessment of arthropod/disease stressors between MON 95379 and the conventional control is meaningful in this study since relatively few pesticides were applied and when they were applied, assessments were conducted before or at least 10 days after pest control measures occurred (with the exception of one protocol deviation), thus minimizing any potential effects of the pesticide treatments.

Table L-2. Summary of Insect Control Measures Applied and Timing of Field Observations of Arthropod Stressors for MON 95379 and the Conventional Control Across in 2018 U.S. Field Trials

Site Code[1]	Date Applied[2]	Commercial Product[3]	Active Ingredient(s)	Application Rate (g a.i./ha)	Carrier Volume (L/ha)[4]	Adjuvant Type	Adjuvant Rate (%)[5]	Application Method	Growth Stage (V to R)	EI Observation Date[2]
ILHY	05/25/2018	Warrior II	Lambda-cyhalothrin	27	140	–	–	On Soil	Pre-emergence	06/20/2018
ILJO	05/17/2018	Force 3G	Tefluthrin	111	–[†]	–	–	In soil	Pre-emergence	06/14/2018
ILJO	07/24/2018	Fastac CS	Alpha-cypermethrin	22	28	–	–	On vegetation	R2	08/06/2018
NEYO[‡]	07/24/2018	Brigade 2EC	Bifenthrin	105	281	Nonionic surfactant	0.3	On vegetation	R2	07/30/2018
NEYO[‡]	07/24/2018	Warrior II	Lambda-cyhalothrin	29					R2	07/30/2018

[1]Site code: ILHY= Clinton County, IL; ILJO= Champaign County, IL; NEYO=York County, NE.
[2]Date format: mm/dd/yyyy.
[3]All product names are the property of their respective owners.
[4]Carrier volume associated with multiple commercial products indicates that the products were applied as a tank mix.
[5]Volume of adjuvant as a percentage of volume of spray solution.
[†]Applied as granule.
[‡]Environmental interaction assessment was done 6 days after insecticide application (instead of 10 days intended) due to oversight (discussed in response to technical review comment 'P. 299, 2nd paragraph').

Table L-3. Summary of Disease Control Measures Applied and Timing of Field Observations of Disease Stressors for MON 95379 and the Conventional Control Across in 2018 U.S. Field Trials

Site Code[1]	Date Applied[2]	Commercial Product[3]	Active Ingredient(s)	Application Rate (g a.i./ha)	Carrier Volume (L/ha)[4]	Adjuvant Type	Adjuvant Rate (%)[5]	Application Method	Growth Stage (V to R)	EI Observation Date[2]
ILJO	07/24/2018	Headline AMP	Pyraclostrobin	107	28	–	–	On vegetation	R2	08/06/2018
			Metconazole	40						
NEYO‡	07/24/2018	Trivapro	Benzovindiflupyr	30	281	Nonionic surfactant	0.3	On vegetation	R2	07/30/2018
			Azoxystrobin	110						
			Propiconazole	125						

[1]Site code: ILHY= Clinton County, IL; ILJO= Champaign County, IL; NEYO=York County, NE.
[2]Date format: mm/dd/yyyy.
[3]All product names are the property of their respective owners.
[4]Carrier volume associated with multiple commercial products indicates that the products were applied as a tank mix.
[5]Volume of adjuvant as a percentage of volume of spray solution.
‡Environmental interaction assessment was done 6 days after insecticide application (instead of 10 days intended) due to oversight (discussed in response to technical review comment 'P. 299, 3rd paragraph').

L.20. Additional Information Regarding Trial Management and Assessment of Abiotic Stressors for Environmental Interaction Assessment (Appendix H)

The Principal Investigator at each site followed local agronomic practices including those related to seed bed preparation and trial maintenance such as application of agricultural chemicals, fertilizer, and irrigation. All maintenance operations were performed uniformly across all plots within each site. In accordance with typical commercial crop production practices, most sites did not receive irrigation and fertilization during the growing season. Only 2 out of 8 sites received irrigation during the growing season. All sites received initial (pre-planting) fertilizer application and only the OHTR site received an in-season fertilizer application. All maintenance operations were performed uniformly across all plots within a site to avoid potential confounding effects from management practices. Therefore, in practice, most sites did not receive irrigation and fertilization during the growing season. However, even though irrigation and fertilizer treatments are implemented, drought and nutrient deficiency could still occur during the season and potentially be influenced by factors such as geographical location, season dynamics and climate. Additionally, the environmental interaction assessment methodology provided flexibility to experienced agricultural researchers to evaluate plants for any relevant stressor that could be present or was actively causing damage to the crop at the time of evaluation at their site. A total of 15 observations for nutrient deficiency were recorded during the season across sites (Table H-3). Seven out of eight sites recorded nutrient deficiency as one of the three abiotic stressors during the growing season. Nutrient deficiency was given a value other than none only in one case so, in practice, nutrient deficiency was not a commonly occurring stressor in these trials. Nutrient deficiency occurred at site ILJO in all six maize entries, consisting of the test MON 95379, the control, and four references at the third environmental interaction evaluation corresponding to R3 growth stage. The degree of severity of damage caused by nutrient deficiency recorded as per the categorical rating scale (categories: none, slight, moderate, or severe) given in section H.6 was slight in test plots and ranged from none to slight in control and reference plots. Although specific symptoms of nutrient deficiency were not recorded, it was likely due to nitrogen and potassium typical for that site and growth stage of the crop as per communication with the field researcher. However, no differences were observed between MON 95379 and the conventional control due to the overlap between the range of injury symptoms between MON 95379 and the conventional control across all four replications within the observation.The remaining stressor assessments from the other six sites had nutrient deficiency with ratings of “none”, which reflects the structure of the environmental interactions assessment methodology.

Field researchers rated for three stressors in each category (insect, disease, and abiotic) that are either actively causing damage or, in the absence of damage symptoms, are typical for that location and time of the season. Including stressors in the absence of damage symptoms adds value as part of a consistent method that contributes to the overall knowledge of what stressors were occurring (or not) at a given site/timepoint combination. Assessment of stressors rated as “none” also provides positive confirmation that the complete assessment was done and facilitates active monitoring of the plots by field researchers.

Therefore, drought and nutrient deficiency are useful to include as potential stressors given that study sites are typically rain-fed and fertilizer application largely occur only prior to planting. In addition, drought and nutrient deificiency are useful stressors to provide flexibility to researchers rating any potential stressor that could be occurring. Furthermore, comparative environmental interaction assessment of drought and nutrient deficiency between MON 95379 and conventional control is meaningful since symptoms from these stressors can still be observed despite use of irrigation and fertilizer treatments.

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L.21. Additional Information Regarding the Threatened and Endangered Species Assessment for MON 95379 Maize (Section VII.B.2 and Section VII.D)

L.21.1. Background

Insect-protected maize MON 95379 produces two insecticidal proteins, Cry1Da_7 and Cry1B.868, which protect against feeding damage caused by targeted lepidopteran insect pests. Cry1Da_7 is a modified Cry1Da protein derived from *Bacillus thuringiensis* (*Bt* subsp. *aizawai*). Cry1B.868 is a chimeric protein comprised of domains I and II from Cry1Be (*Bt*), domain III from Cry1Ca (*Bt* subsp. *aizawai*) and C-terminal protoxin domain from Cry1Ab (*Bt* subsp. *kurstaki*).

MON 95379 was developed to provide growers in South America an additional tool for controlling target lepidopteran pests, including fall armyworm resistant to current *Bt* technologies. MON 95379 will not be commercialized in the United States, but is intended to only be cultivated in small-scale breeding, testing, and seed increase nurseries to develop seed of products that will be sold in other countries, primarily in South America. These intended cultivation uses will be subject to the terms and conditions of an EPA breeding registration, which Bayer has proposed to be limited to no more than 100 acres per growing season across Nebraska, Hawaii, and Iowa.

In July 2020, Bayer submitted a petition for deregulation of MON 95379 to USDA (USDA-APHIS Petition Number: 20-205-01p). Included in the petition, Bayer provided a comprehensive ecological risk assessment (ERA) demonstrating MON 95379 poses minimal risk to organisms beneficial to agriculture, including non-target Lepidoptera, under the proposed conditions of use (e.g., small-scale breeding, testing, and seed increase nurseries). In addition to the ERA, an assessment on the potential effects of MON 95379 on listed threatened and endangered species was conducted considering the proposed conditions of use in a limited geography. Since the results of activity spectrum screening and Tier 1 testing indicated effects on survival from Cry1B.868 and Cry1Da_7 are limited to Lepidoptera, a comparison of the county level distribution of endangered lepidopteran species and corn cultivation was conducted for the three proposed states for cultivation. The results of this county-level overlap analysis identified three species for further evaluation. These species identified were Blackburn's sphinx moth (*Manduca blackburni*) in Hawaii, Dakota skipper (*Hesperia dacotae*) and the Poweshiek skipperling (*Oarisma Poweshiek*) in Iowa and no species in Nebraska. Evaluation of critical habitats and updated ranges for these species supported the conclusion that Blackburn's sphinx moth, Dakota skipper, and Poweshiek skipperling would have no exposure to MON 95379 corn. A recent assessment of potential effects on threatened and endangered species was conducted by the U.S. EPA for MON 89034 ´ TC1507 ´ MON 87411 ´ DAS-59122-7 corn expressing lepidopteran-active Cry1A.105, Cry2Ab2, and Cry1F proteins, resulted in a No Effect determination for all listed lepidopteran species due to habitat requirements that exclude them from corn fields (U.S. EPA, 2017). Updated species range information published by USFWS indicate that Dakota skipper and Poweshiek skipperling have not been observed in Iowa since the time of listing (2014) and are no longer believed to be present in Iowa (USFWS, 2018a; b). Therefore, based on the taxonomic specificity of Cry1B.868 and Cry1Da_7 for Lepidoptera and lack of exposure based on critical habitat and current species range

maps resulted in the conclusion that MON 95379 will have no effect on currently listed threatened and endangered species.

In June 2021, it was communicated to Bayer that as part of the threatened and endangered species assessment for MON 95379, USDA sought additional information on five lepidopteran species. The purpose of this assessment is to provide additional information to support the threatened and endangered species evaluation for five species identified by USDA whose ranges generally overlap with corn-growing regions of the United States. The species evaluated were Mitchell's satyr butterfly (*Neonympha mitchellii mitchellii*), St. Francis' satyr butterfly (*Neonympha mitchellii francisci*), Poweshiek skipperling (*Oarisma poweshiek*), Karner blue butterfly (*Lycaeides melissa samuelis*), and Dakota skipper (*Hesperia dacotae*).

Approach

For this assessment, information is provided to address the identified species collectively as well as individually (Figure L-5). Listed Lepidoptera (i.e., non-pest) species are generally not known to feed on corn tissue as their feeding and reproductive ecology is typically tightly associated with a preferred host plant (U.S. EPA, 2001a). Standard agricultural practices for weed management typically preclude the growth of preferred host plants within the boundaries of corn fields. Therefore, exposure to listed Lepidoptera would only occur by deposition of MON 95379 pollen on preferred host plants adjacent to the field. To refine the qualitative exposure assessment for non-target Lepidoptera provided in the submitted petition for MON 95379, laboratory toxicity information with the most sensitive lepidopteran species tested, monarch butterfly (*Danaus plexippus*), was incorporated with an established pollen deposition model to quantitatively characterize exposure and potential risk at the field edge. Consideration of a proposed product use, and how this use could impact potential exposure, is part of the initial step for developing scenarios to assess risk to listed species (EPA, 2021). The potential for exposure at the field edge was put into context considering standard agronomic practices used to generate high quality seed by limiting pollen flow in seed increase and corn breeding activities. Results and conclusions from the field-edge characterization and product use considerations would apply to all listed Lepidoptera.

The risk assessment for the five species of interest was refined following the general principles outlined in the EPA revised guidance for evaluation of Threatened and Endangered species (EPA, 2020). For each species, county-level comparisons were made between the species ranges provided by the USFWS ECOS database and corn acreage provided in the 2017 USDA Ag census. This overlap analysis was then further refined based on updated state-level and county-level species range information provided in recent 5-Year Reviews and Species Status Updates published by USFWS.

For species such as Karner blue and Mitchell's satyr butterfly, where current species ranges are available only at the county level, no further range refinement was conducted. For St. Francis' satyr butterfly and Poweshiek skipperling, shapefiles downloaded from the USFWS ECOS database were uploaded into USDA CropScape and the percent of the species range occupied by

corn based on the 2020 Crop Data Layers (CDLs) was determined. For Poweshiek skipperling, the current USFWS range map has sub-county species ranges in Michigan and Wisconsin where the species is still present and county-level range information for Iowa, Minnesota, South Dakota, and North Dakota where the species is no longer present. To refine the potential exposure for this species, new polygons were constructed for each individual critical habitat corresponding to the species range in Wisconsin and Michigan and a corn CDL overlay analysis was conducted for each location. The total acres of critical habitat and overlapping corn acres for these sites were then summed to determine the total amount of corn acres overlapping with the most current species range information for Poweshiek skipperling. For the sub-county assessment of Dakota skipper, the current USFWS range map files are too large for USDA CropScape so shapefiles were uploaded into the FESTF Gopher utility (NatureServe and its natural heritage member programs). For the overlap analysis, the current species range and critical habitat information for Dakota skipper was compared to the EPA Use Data Layer for corn. It should be noted that potential exposure and risk is limited to off-field areas due to the lack of host plants within corn fields. Resolution of CDL/UDL-based overlap analyses is typically limited to 30 m and therefore does not provide enough precision to account for the typical off-field transport distances for corn pollen (Pleasants et al., 2001). Therefore, the overlap of the species ranges with the in-field corn CDL/UDLs was determined to be a reasonable, albeit likely highly conservative estimate for potential exposure of listed Lepidoptera to corn pollen.

Following species range refinement, potential exposure of each species was further characterized based on individual species biology and critical habitat considerations. Information for this refinement step was derived from USFWS, NatureServe, and public literature sources.

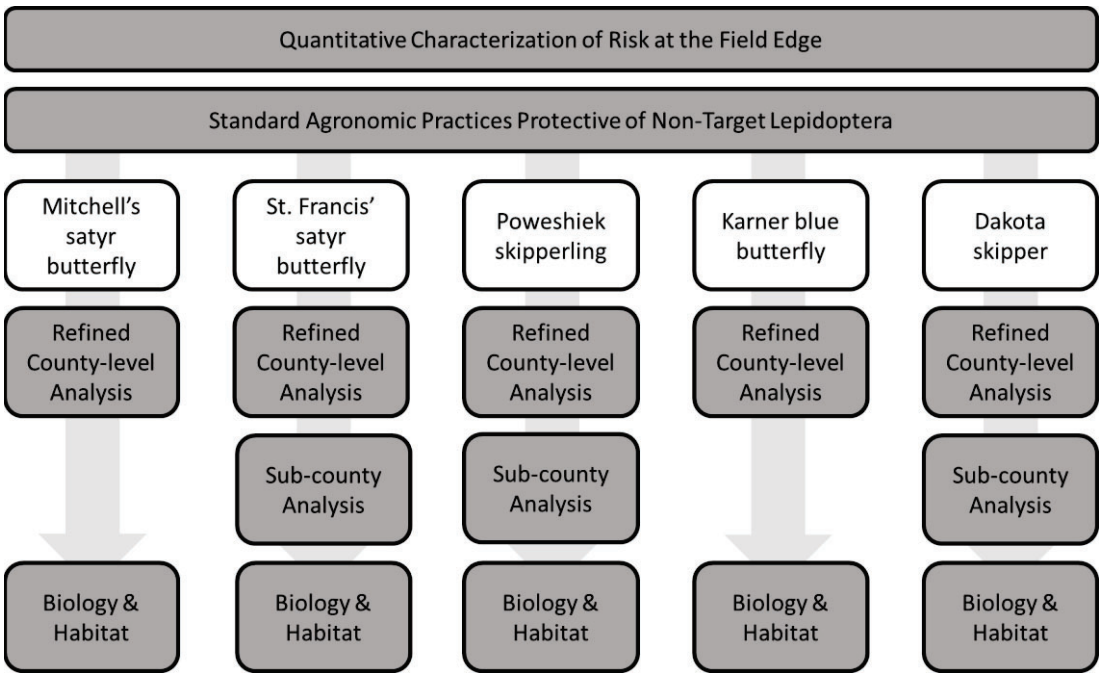

Figure L-5. Framework of the Approach to Provide Geographic, Biological, and Habitat Information to Refine the Threatened and Endangered Species Assessment for MON 95379

L.21.2. Listed Lepidoptera Will Have Minimal Exposure to MON 95379 Corn Pollen

The results of the ecological risk assessment in the submitted petition have shown that the activity of the two *Bt* proteins expressed by MON 95379 are highly specific for lepidopteran insects. Listed Lepidoptera (i.e., non-pest) species are generally known to not feed on corn tissue as their feeding and reproduction is often dependent on a preferred host plant (e.g., wild lupine for Karner blue butterfly and bluestem grasses for Dakota skipper; 2018). Previous work evaluating drift of corn pollen and deposition at the field edge demonstrated that density of pollen deposition on the surface of host plants decreases exponentially with increasing distance from the edge of the field. For example, at a distance of 4 to 5 meters from the edge of a corn field, corn pollen density on host plants is predicted to be less than 10% of the density at the field edge (Pleasants et al. 2001). Consequently, significant exposure of MON 95379 corn pollen to listed Lepidoptera will only occur in areas near the field edge.

In addition, movement of corn pollen is limited to a short interval of time. Peak production of pollen from a corn field typically occurs within a 4-day interval and pollen shed is generally confined to a period of about two weeks (Abendroth et al., 2011). Therefore, sensitive larvae would only be potentially impacted by consumption of MON 95379 pollen if they were feeding on a host plants in close proximity to a MON 95379 field at the time of pollen shed. Rain events that occur during or following pollen shed would significantly reduce pollen densities on host plants near the field. In an analysis of six pollen deposition trials across four regions in the U.S. mainland where corn is cultivated, at least one rainfall event occurred during anthesis in all but one trial and these rain events resulted in 54-86% reduction on deposited pollen densities (Pleasants et al., 2001).

Prevailing wind patterns also influence where pollen deposition will occur in open-pollinated fields. Analysis of prevailing wind patterns in mainland U.S. trials indicated the potential exposure at the field edge from pollen deposition was limited to the downwind side of the field (Pleasants, et al., 2001). Therefore, any habitat present up-wind of a MON 95379 corn field would be expected to have minimal exposure to corn pollen. Additionally, as wind distributes pollen from the field edge, it also disperses the pollen from the surface of host plant leaves where corn pollen has settled and would reduce the expected density of deposition at distances further from the field edge. This would result in further minimizing concentrations of pollen on off-field host plant leaves.

To estimate potential risk of Cry1B.868 and Cry1Da_7 to listed Lepidoptera at the field edge, a quantitative risk analysis was conducted. Cry1Da_7 is not expressed at quantifiable levels in MON 95379 pollen. Therefore, the assessment focused on potential exposure and effects from Cry1B.868. Risk can be conservatively characterized using the U.S. EPA approach for assessing potential risk to endangered animal species and a non-target Lepidoptera risk assessment model incorporating design aspects of confined field trials, such as distance from experimental corn to off-field vegetation.

The U.S. EPA (U.S. EPA. 1992; U.S. EPA, 2004) outlined a conservative approach for assessing potential risk to endangered animal species. For terrestrial animal species, a 10-fold safety factor based on the median lethal concentration (LC_{50}) for the most sensitive species tested is used to accommodate uncertainty in the risk assessment process for the particular terrestrial endangered species. Consequently, a concern for terrestrial endangered animal species would be indicated if the ratio of the LC_{50} concentration for the most sensitive animal species tested to the maximum potential exposure were less than 10. Results from activity screening with Cry1B.868 indicated that monarch butterfly was the most sensitive lepidopteran species tested.

The model developed by Pleasants et al. (2001) and Dively et al. (2004) to assess the risk of *Bt* corn to monarch butterfly larvae was used to determine a safety factor for corn events such as MON 95379 expressing Cry1B.868. The safety factor is derived from model parameters such as toxicity of the protein (i.e., hazard) and exposure considerations such as pollen deposition amounts, and actual amounts of protein encountered by Lepidoptera as they feed on pollen dusted host plant leaves.

The LC_{50} value used for the assessment was determined from results of a laboratory diet-incorporation bioassay with the most sensitive lepidopteran species (monarch butterfly) tested against the Cry1B.868 protein (Table L-4). Exposure to Cry1B.868 from deposition of pollen was calculated for increasing distances from the field edge based on results from published pollen deposition trials (Pleasants et al., 2001).

At a distance of 6 m from the field edge, the margin of safety for Cry1B.868 was calculated to be 10 indicating minimal risk to non-target Lepidoptera (Table L-4). This margin of safety is likely to be highly conservative based on several factors. The estimated exposure levels are derived using 95th percentile values for Cry1B.868 expression as well as 95th-centile values for pollen deposition. This characterization of risk is also highly conservative in that it does not consider host plant distribution in the environment and what proportion of host plants are at the field edge compared to other agricultural and non-agricultural landscapes. The model above is derived from pollen deposition densities on milkweed plants that have horizontally positioned broad leaf architectures and are thus relatively conducive to holding deposited pollen. In contrast, the species of interest feed on grasses (little bluestem for Dakota skipper and Poweshiek skipperling and *Carex* spp. for St. Francis' and Mitchell's satyr butterflies) or wild lupine in the case of Karner blue. The preferred host grasses, discussed further below for each listed species, have relatively narrow (<1cm) leaf blades arranged in a more vertical orientation compared to milkweed leaves and thus would not be expected to collect and maintain significant amounts of corn pollen. Likewise, leaflets of wild lupine are significantly smaller than common milkweed leaves indicating that calculated exposure levels in the milkweed model are likely to considerably overestimate realistic exposure levels.

Table L-4. Estimated Exposure and Hazard as well as the Calculated Margin of Safety for the Cry1B.868 Protein in Pollen Deposited on Host-Plant Leaf Surfaces using Milkweed as a Model System[1].

Distance from edge of the corn field (m)	95th centile pollen deposition on host leaf surface (grains/cm^2)	Maximum dietary exposure for Cry1B.868 (μg/g diet) [2]	7-day LC_{50} for MBF (μg/g diet) [3]	Margin of safety [4]
0	300	0.232	0.076	<1
1	200	0.155	0.076	<1
2	75	0.058	0.076	1
3	54	0.042	0.076	2
4-5	25	0.019	0.076	4
6[5]	10	0.008	0.076	10
7[5]	6	0.004	0.076	17
8[5]	3	0.002	0.076	31

[1] The model used for these calculations is taken, in part, from Pleasants et al., 2001 and Dively et al., 2004. Pollen exposure is based on 95th centile exposure values from Pleasants et al., 2001.

[2] The maximum exposure concentration for both Cry proteins in a pollen-dusted food substrate at a given distance from the edge was calculated by the equation:

{[(a/b)*c]/d}

where:
a = number of the pollen grains deposited per cm2 on the surface of milkweed leaves (surrogate food substrate) at various distances from a cornfield (Pleasants, 2001);
b = number of pollen grains per gram of fresh weight corn pollen [≈ 4 x 10^6 grains according to Miller (1985)];
c = the upper 95th percentile Cry protein concentration of 61.9 μg/g fresh weight: corn pollen;
d = fresh weight (g) of the food plants per cm2 [≈ 0.02 g per cm^2 of fresh milkweed leaves according to the published information in Hellmich et al. (2001)].

[3] For convenience, each mL of diet is assumed to weigh approximately one gram, and therefore the LC_{50} is expressed as μg/g of diet.

[4] The margin of safety was calculated as the quotient of the 7-day LC_{50} value for *D. plexippus* divided by the maximum exposure concentration for the Cry1B.868 protein. For this calculation, the upper 95th percentile Cry1B.868 concentration in pollen of 61.9 μg/g fresh weight was used.

[5] Predicted pollen deposition at 3m and beyond 5 m from the field edge was calculated using regression analysis of distances from field edge and log-transformed deposition values (Pleasants et al., 2001; GraphPad 8 Prism).

There are several examples of commercialized and deregulated corn products cultivated on broad acres with detectable expression of lepidopteran-active proteins in pollen. For example, Cry1A.105 in MON 89034 × TC1507 × MON 88017 × DAS-59122-7 corn has a maximum reported expression of 21 μg/g in pollen (EPA, 2009). Similarly, Cry1F has a maximum reported expression of 35 μg/g in 4114 corn (EPA, 2010) and Vip3A20 in MIR162 corn has reported

maximum expression in pollen of 49.7 µg/g (EPA, 2009). Using a most sensitive species surrogate approach, these products would be expected to have risk profiles at the field edge comparable to MON 95379 (EPA, 2009; Wolt et al., 2005). However, threatened and endangered species conclusions for these products have been largely based on lack of habitat in agricultural areas (EPA, 2009a; 2010; 2017). Consistent with this, USDA concluded MON 89034 corn expressing Cry1A.105 would have no effect on listed Lepidoptera, including specifically St. Francis' satyr butterfly and Mitchell's satyr butterfly, due to a lack of breeding habitat in agricultural areas. (USDA, 2008a). The general lack of overlap between critical habitat and corn fields and the conservative nature of the pollen deposition model indicate cultivation of MON 95379 corn is unlikely to cause adverse effects to listed Lepidoptera under realistic field conditions.

L.21.3. Standard Agricultural Practices to Generate High Quality Seed are Protective of Listed Lepidoptera

Environmental risk mitigation measures are a common consideration in defining conditions of safe use for many pesticides. Standard mitigating measures for MON 95379 corn are derived from typical practices used to limit pollen movement outside of the field and would also be expected to minimize exposure to listed Lepidoptera. A combination of standard agronomic practices (Table L-5) commonly used in seed production and other small-scale breeding and testing nurseries permitted under the proposed EPA registration are expected to further mitigate potential risk to listed Lepidoptera.

Table L-5. Typical Agronomic Practices Expected to Mitigate Off-Field Exposure to Listed Lepidoptera from MON 95379 Pollen During Anthesis.

Agronomic Practice	Mitigating Measure	Benefits
Pre-pollen shed detasseling	Detasseled plants within field prior to pollen shed	Reduces pollen flow off field and eliminates potential exposure to nontarget Lepidoptera habitat.
Border rows	4 to 6 border rows (10 to 15 ft)	Reduces pollen flow off-field to non-target Lepidoptera habitat.
Maintenance of fallow areas surrounding plots	Removal of flowering weeds at the field edge	Reduces Lepidoptera habitat within the field and at field margin and increases distance between field edge and adjacent habitat.
Closed pollination	Bagging of tassels	Limits pollen movement off-field.
Cultivation in controlled environments	Cultivating in greenhouse and screenhouse facilities	Limits pollen flow to non-target Lepidoptera habitat

For example, utilizing a minimum of four rows of conventional corn buffer (10 ft) and a 15 ft fallow area would be protective of non-target Lepidoptera as no effects would be expected at distances >20 ft (~6m) from the field edge under a conservative worst-case exposure scenario (Table L-4). Buffer rows of conventional corn that commonly surround experimental corn plots additionally serve as barriers to pollen flow (Ireland et al., 2006), thus limiting potential deposition on off-field host plants. For these reasons, minimal off-field exposure of non-target Lepidoptera from MON 95379 corn pollen is expected and this exposure is further minimized by standard agronomic practices used to generate high quality seed with activities permitted under the proposed EPA registration.

L.21.4. Species-Specific Information to Support the Threatened and Endangered Species Assessment

a. Mitchell's satyr butterfly

Species Range

The current range for Mitchell's satyr butterfly (MSB) is limited to counties within the states of Alabama, Indiana, Michigan, Mississippi, Ohio, and Virginia (Table L-6). While the current species range overlaps counties with corn production, MSB is not present in Hawaii, Iowa, or Nebraska where MON 95379 is intended for cultivation.

Table L-6. County-Level Overlap of Corn Acres with Mitchell's Satyr Butterfly[a]

State	County
Alabama	Bibb, Greene, Hale, Perry, Tuscaloosa
Indiana	LaGrange
Michigan	Barry, Berrien, Branch, Cass, Jackson, Kalamazoo, St. Joseph, Van Buren, Washtenaw
Mississippi	Itawamba, Monroe, Prentiss
Ohio	Portage
Virginia	Floyd

[a] County-level species range derived from USFWS ECOS (accessed 8/10/2021). County-level corn acres derived from USDA AgCensus, 2017.

Species range refinement

MSB is currently believed to exist in 9 counties between Michigan and Indiana in fens ranging in size between 0.5 and 130Ha for a total of 504 Ha. At the time of listing, MSB was known to exist in a single county in Ohio, but no extant populations were identified in surveys spanning 1985-1990 and as of 2013, the species is believed to be extirpated from Ohio. Twenty-eight sites are currently occupied by MSB in Alabama, most within the protected lands of the Oakmulgee Ranger District of the Talledega National Forest. Previous surveys identified 11 extant colonies withing the protected Natchez Trace Parkway in Mississippi and 17 sites within the Blue Ridge Mountains region of Virginia (USFWS, 2021b).

The range maps for MSB provided by USFWS are at the county level and thus, no further refinement of the species range was conducted (Figure L-6).

Biology and habitat

The life cycle of MSB consists of a single annual brood where hatched larvae feed through the summer before entering diapause through the winter and resume feeding the following spring. In late May to early June, larvae form a chrysalis and adults emerge mid-June through late July. While females may oviposition on a variety of plant species, hatched larvae migrate to food plants including those that do not support full development. Full development and maturation are believed to be supported by the preferred host plant, *Carex stricta*, a sedge that serves as the primary food plant (USFWS, 2021b).

C. stricta is restricted to sedge meadows, shallow marshes, fens, shores, streambanks, and ditches, consistent with the known habitat for MSB:

"Although MSB habitat requirements are not yet fully understood, the butterfly appears to be restricted to calcareous wetlands that range along a continuum from open fen, wet prairie, sedge meadow, shrub-carr, tamarack savanna, and numerous variations and combinations of these community types (Shuey 1997, Szymanski 1999, Hyde et al. 2001). It appears that the MSB occupies areas in these fen communities where woody and herbaceous vegetation occurs as a mosaic (Szymanski and Shuey 2002). Important structural components of the habitat include presence of peat or muck soil (Shuey 1997), scattered deciduous shrubs or coniferous trees (Shuey 1997), seeps (McKinnon and Albert 1996) and a herbaceous community dominated by C. stricta. MSB habitat also appears to exhibit large variability in vegetative structure and composition at the habitat patch scale, suggesting the importance of habitat heterogeneity (Szymanski 1999). Recent research has further reinforced the importance of the edge component; in the later part of the adult flight period (i.e., during the time of oviposition), males and females tend to be found within one meter of a tree or shrub (Barton 2003)."

-NatureServe, 2018

The perennial sedge, *Carex stricta,* also known as an upright or hummock sedge, is typically found in sedge meadows, shallow marshes, fens, shores, streambanks, and ditches. Leaves are typically 2-6 mm in width (USDA, 2008b) extending relatively vertically from the base of the plant. Based on the plant architecture and relatively narrow leaf blades, levels of corn pollen retained on the leaf surface would be expected to be far less than those observed with common milkweed or artificial pollen traps (Pleasants et al., 2001) thus further minimizing potential exposure to MSB larvae.

While MSB populations can be found in counties that have corn production, MSB exhibits preference for sedge grasses and wetland habitats that are not expected to be amenable to corn production. Furthermore, several of the populations currently exist within protected lands. Consistent with this, USDA concluded for MON 89034 corn that there would be no effect on MSB due to a lack of proximity of habitat to cultivated corn (USDA, 2008a). Given the habitat and host plant preferences of MSB, no significant exposure to MON 95379 pollen is expected.

b. St. Francis' satyr butterfly

Species Range

The current range for St. Francis' satyr butterfly (SFSB) is limited to counties within the state of North Carolina (Table L-7). While the current species range overlaps counties with corn production, the species is not present in Hawaii, Iowa, or Nebraska where MON 95379 is intended for cultivation.

Table L-7. County-level overlap of corn acres with St. Francis' satyr butterfly[a]

State	County
North Carolina	Cumberland, Harnett, Hoke, Moore

[a]County-level species range derived from USFWS ECOS (accessed 8/10/2021). County-level corn acres derived from USDA AgCensus, 2017.

Species Range Refinement

As of 2020, no populations have been observed outside of Department of Defense lands at the Ft. Bragg Army installation since the time of listing despite extensive surveys. To confirm anticipated minimal exposure of SFSB to cultivated corn, an overlap analysis was conducted at the sub-county level using the refined current USFWS species range for SFSB (Figure L-7). The landscape of the current SFSB range is predominantly forest, woody wetlands, and developed land covers associated with the military installation. Analysis of the CDLs within the species range indicate that corn represents approximately 0.3% of the land use and is limited to the very outer edges of the expected species range (Table L-8 Figure L-7). The results of the analysis confirm minimal overlap between the SFSB species range and cultivated corn.

Table L-8. Refined Overlap of the Corn CDL and the Current Species Range of SFSB

CDL ID Value	Category	Acreage
142	Evergreen Forest	124908.4
190	Woody Wetlands	20729.2
121	Developed/Open Space	18156.7
122	Developed/Low Intensity	12096.7
131	Barren	10606.9
152	Shrubland	10075.4
176	Grass/Pasture	3181.4
141	Deciduous Forest	2435.9
123	Developed/Medium Intensity	2039.1
143	Mixed Forest	2035.1
5	Soybeans	1662
37	Other Hay/Non Alfalfa	1615.5
111	Open Water	1584.1
1	**Corn**	**687.2**
61	Fallow/Idle Cropland	578.2
2	Cotton	116.5
195	Herbaceous Wetlands	106.7
124	Developed/High Intensity	79.2
59	Sod/Grass Seed	75.8
26	Dbl Crop WinWht/Soybeans	71.8
27	Rye	34.2
24	Winter Wheat	31.6
44	Other Crops	24
29	Millet	18
4	Sorghum	12.9
28	Oats	7.1
10	Peanuts	5.6
53	Peas	5.6
46	Sweet Potatoes	5.1
67	Peaches	4.2
11	Tobacco	3.1
69	Grapes	3.1
242	Blueberries	1.3
47	Misc Vegs & Fruits	1.1
48	Watermelons	0.9
74	Pecans	0.9
50	Cucumbers	0.7

237	Dbl Crop Barley/Corn	0.4
205	Triticale	0.2
221	Strawberries	0.2
226	Dbl Crop Oats/Corn	0.2
236	Dbl Crop WinWht/Sorghum	0.2
254	Dbl Crop Barley/Soybeans	0.2
	Total	**213002.6**

Biology and habitat

The life cycle of the St. Francis' satyr butterfly (SFSB) consists of two brood cycles per season where the first flight adults emerge late May to early June and then again in July through early August. The last brood overwinters in the larval stage before resuming feeding and completing development in the spring. While caterpillars have been difficult to identify in surveys, the two that have been found have been associated with the presumed preferred host plant sedge, *Carex mitchelliana*. The species is generally sedentary, and any movement would be expected to be between proximal subpopulations (USFWS, 2020).

The preferred habitat for SFSB is described as:

> *"The habitat occupied by this satyr consists primarily of wide wet meadows dominated by a high diversity of sedges (Carex spp.) and other wetland graminoids. In the North Carolina sandhills, such meadows are often relicts of beaver activity. Saint Francis' satyr has also been observed in pitcher plant (Sarracenia flava) swales, with cane (Arundinaria tecta), and with the rare plants rough-leaved loosestrife (Lysimachia asperulaefolia, federally listed as endangered) and pocosin lily (Lilium iridollae, a species of Federal concern). It is, however, unknown whether the satyr uses such swale habitat for feeding, breeding, and perching, or simply as a dispersal corridor. Unlike the habitat of Mitchell's satyr, the North Carolina species' habitat cannot properly be called a fen because the waters of this sandhills region are extremely poor in inorganic nutrients (USFWS, 1996). Known only from a few sedge wetlands in close proximity. Habitat apparently open seepage areas dominated with Carex. Habitat is successional or disclimax with both beaver and fires being apparently critical factors in maintaining it (Natureserve, 2015). Clumped spatial arrangement and narrow environmental specificity are based on specific habitat requirements of the species as are high ecological integrity and site fidelity and low tolerance range. (USFWS, 1996; NatureServe, 2015)."*
>
> -NatureServe, 2015

The preferred host plant for SFSB larvae is not currently known. Surveys and limited captive rearing have shown SFSB larvae to feed on *Carex spp.* including *Carex mitchelliana* and *Carex*

atlantica. USFWS notes that it is possible for larvae to feed on more than one host plant over the course of each year due to the fact that *C. mitchelliana* senesces relatively later in the summer compared to other *Carex* species (USFWS, 2020). *Carex spp.* leaves are typically 2-6 mm in width and extend relatively vertically from the base of the plant (USDA, 2008b). Based on the plant architecture and relatively narrow leaves, levels of deposited and retained pollen on the leaf surface would be expected to be far less than those observed with common milkweed or artificial pollen traps (Pleasants et al., 2001), thus further minimizing potential exposure to SFSB larvae.

While SFSB populations can be found in counties that have corn production, SFSB exhibits preference for sedge grasses and wetland habitats that are not expected to be amenable to corn production. Furthermore, all populations currently exist within DoD lands with minimal overlap to commercial corn cultivation. Consistent with this, USDA concluded for MON 89034 corn that there would be no effect on SFSB due to a lack of proximity of habitat to cultivated corn (USDA, 2008a). Considering critical habitat and host plant preferences of SFSB, no significant exposure to MON 95379 pollen is expected.

c. Poweshiek skippering

Species Range

The range for Poweshiek skipperling (PS) provided by a query of the USFWS ECOS database is limited to counties within the states of Iowa, Michigan, Minnesota, North Dakota, South Dakota, Wisconsin (Table L-9).

Table L-9. County-level overlap of corn acres with Poweshiek skipperling[a]

State	County
Iowa	Cerro Gordo, Dickinson, Emmet, Hancock, Howard, Kossuth, Osceola
Michigan	Hillsdale, Jackson, Lenawee, Livingston, Oakland, Washtenaw
Minnesota	Polk
North Dakota	Richland, Sargent
South Dakota	Brookings, Clark, Codington, Day, Deuel, Grant, Marshall, Roberts
Wisconsin	Green Lake, Waukesha

[a] County-level species range derived from USFWS ECOS (accessed 8/10/2021). County-level corn acres derived from USDA AgCensus, 2017.

Species Range Refinement

As part of the 2018 5-year review published by USFWS for the Poweshiek skipperling, the updated species range for each state identified in the ECOS database is as follows:

Michigan:

> *"All six sites where the Poweshiek skipperling was considered extirpated at the time of listing are still considered extirpated…Five Michigan sites are currently classified as present, out of the nine that had present status at the time of listing. However, the numbers of individuals detected at these remaining present sites have decreased since listing, with high daily counts of 1, 2, and 9 individuals in 2018 at Halstead Lake Fen, Holly Fen (Brandt Road), and Buckhorn Lake (Big Valley), respectively. The stronghold of Long Lake Fen (2 sites, including the Eaton Road site) has not had a high daily count above 49 since listing, with a high daily count of 28 in 2018 (compared to multiple counts in the hundreds in the five years preceding listing). Similarly, numbers are down for the maximum number of Poweshiek skipperlings observed per minute at these sites relative to the recent years before listing (Figure L-5). Belitz and colleagues (2019, p. 645) estimated the adult Poweshiek abundance in Michigan prairie fens to be 231 (95% CI 160-332). Furthermore, no additional sites have been found, even though a habitat model identified approximately 33 sites that may have significant potential to be inhabited by Poweshiek skipperling. Of the potential sites surveyed thus far, no new Poweshiek skipperling sites have been found (MNFI 2017, unpublished)."*
>
> -USFWS, 2018b

Wisconsin:

> *"At the time of listing, there were three sites with unknown occupancy and one site where Poweshiek skipperling were present. The three sites with previously unknown occupancy are now all considered extirpated. The site with Poweshiek skipperling presence, Puchyan Prairie, is still considered to be present. Since 2012, no more than three Poweshiek skipperlings have been observed in a given year at that site. In both 2017 and 2018, there was one individual sighted, however no photo documentation confirms these sightings."*
>
> -USFWS, 2018b

Minnesota:

> *"Poweshiek skipperling was once widespread and abundant in Minnesota; however there have been no confirmed sightings of the species in the state since 2007 (U.S. Fish and Wildlife Service 2019, unpub. data). One unconfirmed sighting in 2013 occurred at a prairie complex owned and managed primarily by the Minnesota Department of Natural Resources (MNDNR) in the Chicog Wildlife*

Management Area (WMA). This area has had recent adult observations over multiple years (2004-2007, and unconfirmed in 2013). Follow-up surveys since in 2014 and 2016 resulted in no detections of the species at Chicog WMA (MNDNR 2017, unpub. data)."

-USFWS, 2018b

Iowa, North Dakota, South Dakota:

"Since the time of listing, there have been no sightings in Indiana, Illinois, Iowa, North Dakota, and South Dakota. There are no sites where the Poweshiek skipperling is currently considered present in those states."

-USFWS, 2018b

Since the Minnesota observations are unconfirmed and located within wildlife management areas, additional refinement on the current range for PS was conducted by focusing on the two states where PS is still considered present: Michigan and Wisconsin. The current species range was recently confirmed in a published draft Biological Opinion for Malathion that indicates PS is now only present in two states (USFWS, 2021). The species range maps for Michigan and Wisconsin correspond to areas designated as critical habitat in these states. The 2018 5-year review was used to screen for critical habitat units within Michigan and Wisconsin where PS was still believed to be present (Table L-10).

Table L-10. Locations of Current Species Range for PS in Michigan and Wisconsin

State	Unit	Name	Status[1]
MI	1	Holly Fen	Present
MI	2	Halstead Lake	Present
MI	3	Long Lake	Present
MI	4	Buckhorn Lake	Present
MI	5	Bullard Lake	Extirpated
MI	6	Park Lydon	Unknown
MI	7	Goose Creek	Unknown
MI	8	Liberty Fen	Extirpated
MI	9	Liberty Bowl Fen	Possibly Extirpated
WI	1	Waukesha Co	Present
WI	2	. Green Lake Co.	Extirpated

[1]Current species statues provided by USFWS in the 2018 5-year review for Poweshiek skipperling (USFWS, 2018b).

To quantitatively refine the potential overlap between corn acres and the current PS species range, each critical habitat unit where PS was either present, unknown, or possibly extirpated was queried within the USDA CropScape against the 2020 CDLs (Figures L-8 – L-16). When the CDL layers were evaluated, the total corn acres accounted for 0.3% of the critical habitat acres in Michigan and Wisconsin indicating minimal exposure of PS to cultivated corn (Table L-11).

Table L-11. Refined Overlap of the Corn CDL and the Current Species Range of PS

State	Unit	CDL ID Value	Category	Acreage
MI	1	121	Developed/Open Space	1.1
MI	1	141	Deciduous Forest	5.6
MI	1	190	Woody Wetlands	19.6
MI	**2**	**1**	**Corn**	**0.2**
MI	2	61	Fallow/Idle Cropland	0.2
MI	2	111	Open Water	3.6
MI	2	121	Developed/Open Space	0.7
MI	2	141	Deciduous Forest	4.2
MI	2	143	Mixed Forest	0.4
MI	2	190	Woody Wetlands	60.7
MI	2	195	Herbaceous Wetlands	1.3
MI	**3**	**1**	**Corn**	**1.3**
MI	3	5	Soybeans	0.7
MI	3	36	Alfalfa	0.2
MI	3	61	Fallow/Idle Cropland	2
MI	3	111	Open Water	50.3
MI	3	121	Developed/Open Space	4.2
MI	3	122	Developed/Low Intensity	1.3
MI	3	141	Deciduous Forest	79.4
MI	3	143	Mixed Forest	0.4
MI	3	176	Grass/Pasture	2.7
MI	3	190	Woody Wetlands	258.9
MI	3	195	Herbaceous Wetlands	2.4
MI	**4**	**1**	**Corn**	**0.9**
MI	4	4	Sorghum	0.2
MI	4	61	Fallow/Idle Cropland	1.1
MI	4	111	Open Water	15.1
MI	4	121	Developed/Open Space	2.9
MI	4	122	Developed/Low Intensity	0.2
MI	4	141	Deciduous Forest	48
MI	4	176	Grass/Pasture	4.4
MI	4	190	Woody Wetlands	198.2
MI	4	195	Herbaceous Wetlands	1.1
MI	6	61	Fallow/Idle Cropland	0.2
MI	6	111	Open Water	12.7
MI	6	141	Deciduous Forest	26
MI	6	143	Mixed Forest	0.7
MI	6	176	Grass/Pasture	9.3
MI	6	190	Woody Wetlands	211.5

MI	6	195	Herbaceous Wetlands	0.9
MI	**7**	**1**	**Corn**	**1.3**
MI	7	5	Soybeans	0.7
MI	7	61	Fallow/Idle Cropland	1.1
MI	7	111	Open Water	0.2
MI	7	121	Developed/Open Space	0.7
MI	7	122	Developed/Low Intensity	0.9
MI	7	141	Deciduous Forest	2.4
MI	7	143	Mixed Forest	0.2
MI	7	176	Grass/Pasture	4
MI	7	190	Woody Wetlands	111.4
MI	7	195	Herbaceous Wetlands	0.2
MI	9	141	Deciduous Forest	0.9
MI	9	190	Woody Wetlands	33.8
WI	2	141	Deciduous Forest	6.9
WI	2	143	Mixed Forest	0.2
WI	2	190	Woody Wetlands	2.2
WI	2	195	Herbaceous Wetlands	188.6
			Total	**1390.5**

Biology and habitat

PS have a single flight per year that occurs middle of June through the middle of July. The flight period for PS individuals lasts one to two weeks where mating and egg laying occurs. Eggs typically hatch late summer whereby larvae feed and develop through early fall. Larvae then overwinter before completing development in the spring.

The historical habitat for PS has been described as prairie grasslands and meadows where there preferred host plants would be found. Only 4% of the original native tall grass prairies remain in small isolated sites that limit movement of PS. Presently, the known remaining PS populations are present on either protected conservation lands or wetland environments in Michigan and Wisconsin.

> *"The disjunct populations of Poweshiek skipperlings in Michigan have more narrowly defined habitat preferences, variously described as wet marshy meadows (Holzman 1972, p. 114), bog fen meadows or carrs (Shuey 1985, p. 181), sedge fens (Bess 1988, p. 13), and prairie fens (Michigan Natural Features Inventory 011, unpubl. data; Michigan Natural Features Inventory 2012, unpubl. data). Bess (1988, p. 13) found the species primarily in the drier ortions of Liberty Fen, Jackson County, dominated by ''low sedges'' and an abundance of nectar sources. Summerville and Clampitt (1999, p. 231) noted that the population was concentrated in areas dominated by spikerush and that only 10–15 percent of the fen area was occupied despite the abundance of nectar sources throughout. Poweshiek skipperling have been described as occupying peat domes within larger prairie fen complexes in areas either dominated by mat muhly or prairie dropseed (Cuthrell 2013a, pers. comm.)… Puchyan Prairie consists of wet-mesic prairie that*

grades lower into sedge meadow (WI DNR Web site http://dnr.wi.gov/topic/ Lands/naturalareas/ index.asp?SNA=172; Swengel 2013, pers. comm.) and adult Poweshiek Skipperlings have been observed in wet prairie there, although it is not known if these areas function as successful larval habitat (Swengel 2013, pers. comm.). Like the Dakota skipper, it has been hypothesized that Poweshiek skipperling larvae may be vulnerable to desiccation during dry summer months (Borkin 2012a, pers. comm.) and require movement of shallow groundwater to the soil surface or wet low areas to provide relief from high summer temperatures or dry conditions (Royer et al. 2008, pp. 2, 16; Borkin 2012a, pers. comm.).”

-NatureServe, 2021

As described above, the habitats represented in the current range of PS are described principally as fens or wetland prairies, which would not be suitable for cultivation of corn, and this conclusion is consistent with the results of the refined overlay analysis.

Larvae feed on native grass species, however little bluestem (*Schizachyrium scoparium*) is a preferred food source. Grass species such as little bluestem provide the larvae with a dense cluster of erect blades in proximity to an abundance of edible leaf tissue (USFWS, 2018b). Little bluestem is a warm-season perennial grass with broad distribution in the U.S. and Canada. Leaf blades are relatively vertically positioned, 5-30 cm long and 1.5-6 mm wide (USDA, 2013). Based on the plant architecture and relatively narrow leaves, levels of deposited and retained pollen on the leaf surface would be expected to be far less than those observed with common milkweed or artificial pollen traps (Pleasants et al., 2001), thus further minimizing potential exposure to PS larvae.

While PS populations can be found in counties with reported corn acreage, the current known species range is limited to Michigan and Wisconsin in wetland habitats that are not expected to be in close proximity to corn production. Therefore, given the current species range, preferred habitat and host plant specificity, no significant exposure to MON 95379 pollen is expected.

d. Karner blue butterfly

Species Range

The current range for Karner blue butterfly (KBB) is limited to counties within the states of Illinois, Indiana, Michigan, Minnesota, New Hampshire, New York, Ohio, and Wisconsin (Table L-12). Based on the 2017 AgCensus, no corn acres were produced for grain in Merrimack county, NH. Merrimack county is the only county in NH where KBB is believed to be present and thus the state was excluded from the county-level overlap table. While the current species range overlaps in counties with corn production at the national level, the species is not currently found in Hawaii, Iowa, or Nebraska where MON 95379 is intended for cultivation.

Table L-12. County-Level Overlap of Corn Acres with Karner Blue Butterfly[a]

State	County
Illinois	Lake
Indiana	Lake, Porter
Michigan	Allegan, Ionia, Kent, Lake, Mason, Mecosta, Monroe, Montcalm, Newaygo, Oceana
Minnesota	Winona
New York	Albany, Saratoga, Schenectady
Ohio	Lucas
Wisconsin	Adams, Barron, Burnett, Chippewa, Clark, Columbia, Dane, Dunn, Eau Claire, Green Lake, Iowa, Jackson, Marquette, Menominee, Monroe, Oconto, Portage, Richland, Sauk, Shawano, St. Croix, Vernon, Waupaca, Waushara, Wood

[a]County-level species range derived from USFWS ECOS (accessed 8/10/2021). County-level corn acres derived from USDA AgCensus, 2017.

Species range refinement

In the 2019 5-year review for KBB, USFWS notes of the 8 states with KBB at the time of listing in 1992, KBBs are likely no longer present in Illinois, Minnesota, and Indiana (USFWS, 2019). The current range maps for this species are at the county level for the largest population in Wisconsin and therefore, no sub-county refinement could be conducted (Figure L-17).

Biology and habitat

The potential for risk to KBB as a result of exposure to *Bt* corn has been previously assessed (Mendelsohn et al., 2003; Peterson et al., 2006). KBBs are found along the northern range of wild lupine, comprising parts of Wisconsin, Michigan, Minnesota, Indiana, New Hampshire and New York where packed snow occurs late into the spring (Peterson et al., 2006; US EPA, 2001b; USFWS, 2003). Wisconsin supports the largest population of KBB, which is found in 25 Wisconsin counties. There are two complete generations of KBB per year. Larvae from the first-generation hatch from eggs in mid-April, well before corn pollen shed in these geographic areas. The adults emerge in late May to early June. Larvae from the second-generation feed through mid-July and the adults emerge through mid-August. Eggs from the second generation are the overwintering stage.

Wild lupine is the only known host for KBB larvae. Wild lupine grows on dry, sandy soils in pine barrens, oak savannahs, and disturbed habitats such as forest trails, roadsides, military training

areas, airports, highway corridors, sand roads and abandoned sand pits (USFWS, 2003; Peterson et al., 2006). The preferred habitat of KBB is best described as broken or scattered tree canopies that vary from 0 to 50% and up to 80% canopy cover, with a mixture of grasses and forbs in the open areas. Compared to milkweed, the leaflets of wild lupine are relatively small; typically 2" long and only half an inch wide (Minnesota Wildflowers, 2021). Therefore, the pollen deposition rates based on milkweed leaves is likely a highly conservative estimate of exposure to KBB larvae feeding on wild lupine. Habitat destruction, through development and land practices, is the major cause of the decline of KBB populations (USFWS, 2003).

Wild lupine does not occur in corn fields. Data from Peterson et al. (2006) show that <10% of all lupine sites are even within 100 meters of a corn field and indeed, approximately 80% of all lupine sites are more than 500 meters from a corn field. There is also evidence that many extant KBB populations are spatially separated from production agriculture areas by large bodies of water, urban areas or publicly owned land. \ The Wisconsin Department of Natural Resources has concluded: "Most agricultural operations do not appear to support habitat for the Karner blue butterfly or present a threat to the continued existence or recovery of the Karner blue butterfly in Wisconsin." (Wisconsin Dept. Natural Resources, 2010). This assertion is supported by the recent work of Peterson et al. (2006).

KBB larvae use wild lupine exclusively as a host plant; therefore, the only route of exposure of Cry1B.868 and Cry1Da_7 to Karner blue larvae is through deposition of pollen from corn MON 95379 onto lupine plants (USFWS, 2003; Peterson et al., 2006). In order for KBB larvae to be exposed to pollen from MON 95379, lupine plants must first be in and around corn fields and, more importantly, pollen shed must occur onto the lupine at the time KBB larvae are feeding. In the northern corn maturity zones where KBB is present, corn plants (field corn, sweet corn or popcorn) typically shed pollen after mid to late July or early August (USFWS, 2003). Thus, even if wild lupine plants occurred in or close to corn fields, it is highly unlikely that corn pollen would be present at the time KBB larvae are actively feeding. Degree-day effects that might result in earlier or later pollen shed would also tend to correspondingly affect the rate of KBB development, and therefore not contribute to any greater likelihood of *Bt* pollen exposure. An analysis based on degree-day modeling of KBB butterfly phenology and corn pollen shed (Peterson et al., 2006) showed that there are only two Wisconsin locations (counties) in the Midwestern corn belt where there is a potential temporal overlap between KBB larvae and corn pollen shed. However, there was no evidence that KBB larvae were exposed to corn pollen in these two locations (Peterson et al., 2006). Based on the lack of overlap of critical habitat and host plants in proximity to corn fields, significant exposure of MON 95379 to KBB is not anticipated.

e. Dakota skipper

Species Range

The current range for the Dakota skipper (DS) is limited to counties within the states of Minnesota, North Dakota, and South Dakota (Table L-13). While the current species range overlaps in counties with corn production, the species is not currently found in Hawaii, Iowa, or Nebraska where MON 95379 is intended for cultivation.

Table L-13. County-Level Overlap of Corn Acres with Dakota Skipper[a]

State	County
Minnesota	Big Stone, Chippewa, Clay, Lac qui Parle, Lincoln, Norman, Pipestone, Stevens, Swift, Traverse
North Dakota	Barnes, Benson, Billings, Bottineau, Burke, Burleigh, Cass, Dickey, Divide, Dunn, Eddy, Emmons, Foster, Griggs, Kidder, Lamoure, Logan, McHenry, McKenzie, McLean, Mercer, Morton, Mountrail, Nelson, Oliver, Pierce, Ramsey, Ransom, Renville, Richland, Rolette, Sargent, Sheridan, Stark, Steele, Stutsman, Traill, Ward, Wells, Williams
South Dakota	Brookings, Brown, Clark, Codington, Day, Deuel, Edmunds, Grant, Hamlin, Kingsbury, Lake, Marshall, McCook, McPherson, Minnehaha, Moody, Roberts, Spink, Turner

[a]County-level species range derived from USFWS ECOS (accessed 9/22/2021). County-level corn acres derived from USDA AgCensus, 2017.

Species range refinement

The states of Minnesota, North Dakota, and South Dakota contain the only known remaining populations of Dakota skipper in the United States. An overlap analysis was conducted with the UDL for corn and the critical habitat and species range for DS within the FESTF Gopher utility. Based on the results of the overlap analysis, 12.7% of the species range and 2.2% of the DS critical habitat overlaps with corn acres (Figure L-18 and L-19).

Biology and habitat

DS have a single flight per year that occurs middle of June through the end of July. The flight period for DS individuals lasts two to four weeks where mating and egg laying occurs. Flight distances are relatively limited as DS are believed to not travel more than 1km between habitat patches. Eggs are laid on broadleaf plants and grasses. Eggs typically hatch within 14 days whereafter larvae crawl to the base of grass plants at the soil surface. Larvae develop to 4th or 5th instars where they then overwinter before completing development in the spring.

Larvae feed on native grass species, but little bluestem (*Schizachyrium scoparium*) is a preferred food source. Grass species such as little bluestem provide the larvae with a dense cluster of erect blades in proximity to an abundance of edible leaf tissue (USFWS, 2018a). Little bluestem is a warm-season perennial grass with broad distribution in the U.S. and Canada. Leaf blades are relatively vertically positioned, 5-30 cm long and 1.5-6 mm wide (USDA, 2013). Based on the plant architecture and relatively narrow leaves, levels of deposited and retained pollen on the leaf surface would be expected to be far less than those observed with common milkweed or artificial pollen traps (Pleasants et al., 2001), thus further minimizing potential exposure to DS larvae.

DS habitat can be classified into one of two types. Type A habitat consists of low wet-mesic prairie that occurs on near-shore glacial lake deposits. This type of habitat common in North Dakota and parts of South Dakota may be subject to flooding and is dominated by bluestem grasses. The second type of habitat (Type B) can be found in Minnesota and occurs on rolling terrain over gravelly glacial moraine deposits. Present-day populations of DS are now found in remnant native-prairie habitats that have soils typically unsuitable for agriculture and steep topography (USFWS, 2018a). Both factors would be expected to significantly limit exposure of DS larvae to cultivated corn fields and is consistent with the minimal overlap between DS critical habitat and corn acres. Therefore, significant exposure of DS to MON 95379 is not anticipated.

L.21.5. Conclusion

Under the proposed EPA registration, cultivation of MON 95379 is limited to a combined total of 100 acres per growing season in Nebraska, Hawaii, or Iowa. An overlay analysis at the county level comparing areas of corn cultivation with the most current species range information for the five listed lepidopteran species of interest confirm that these species are not present within the intended states of cultivation. Quantitative characterization of risk at the field edge indicate adverse effects would not be anticipated outside at distances of 6 m or greater from the field edge, but it should be noted that this characterization relied on highly conservative assumptions for protein expression, pollen deposition, and landscape distribution of host plants. The conservativeness of this assessment is further demonstrated by characterization of preferred host plant architectures indicating these host plants are much less likely to retain deposited pollen compared to common milkweed used in the field-edge characterization model. The results of the field-edge assessment when placed into context of the intended product use indicate that typical agronomic practices used to generate high quality seed in breeding and seed increase activities permitted under the proposed EPA registration would limit off-field pollen flow and thus be protective of listed Lepidoptera. Analyses of current species range maps, species biology and critical habitats support the conclusion that these species occupy habitat landscapes distinct from areas of corn production. Therefore, the above considerations in addition to the intended use for MON 95379 corn are expected to be protective of listed Lepidoptera.

L.21.6. Refined Species Range Maps

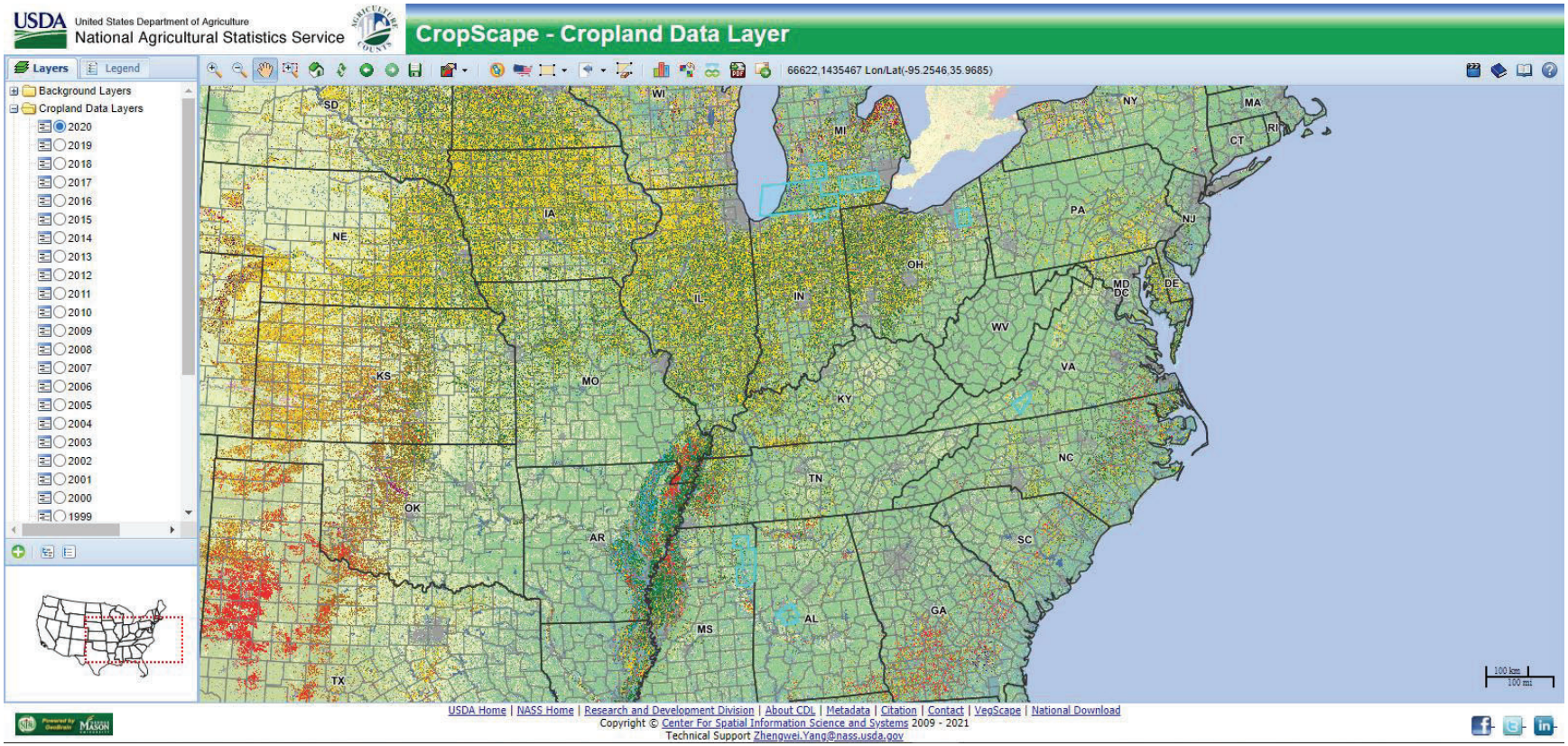

Figure L-6. Current Species Range for MSB (accessed 9/27/2021)

Current species range is indicated by blue polygons and the CDL for corn is indicated in yellow.

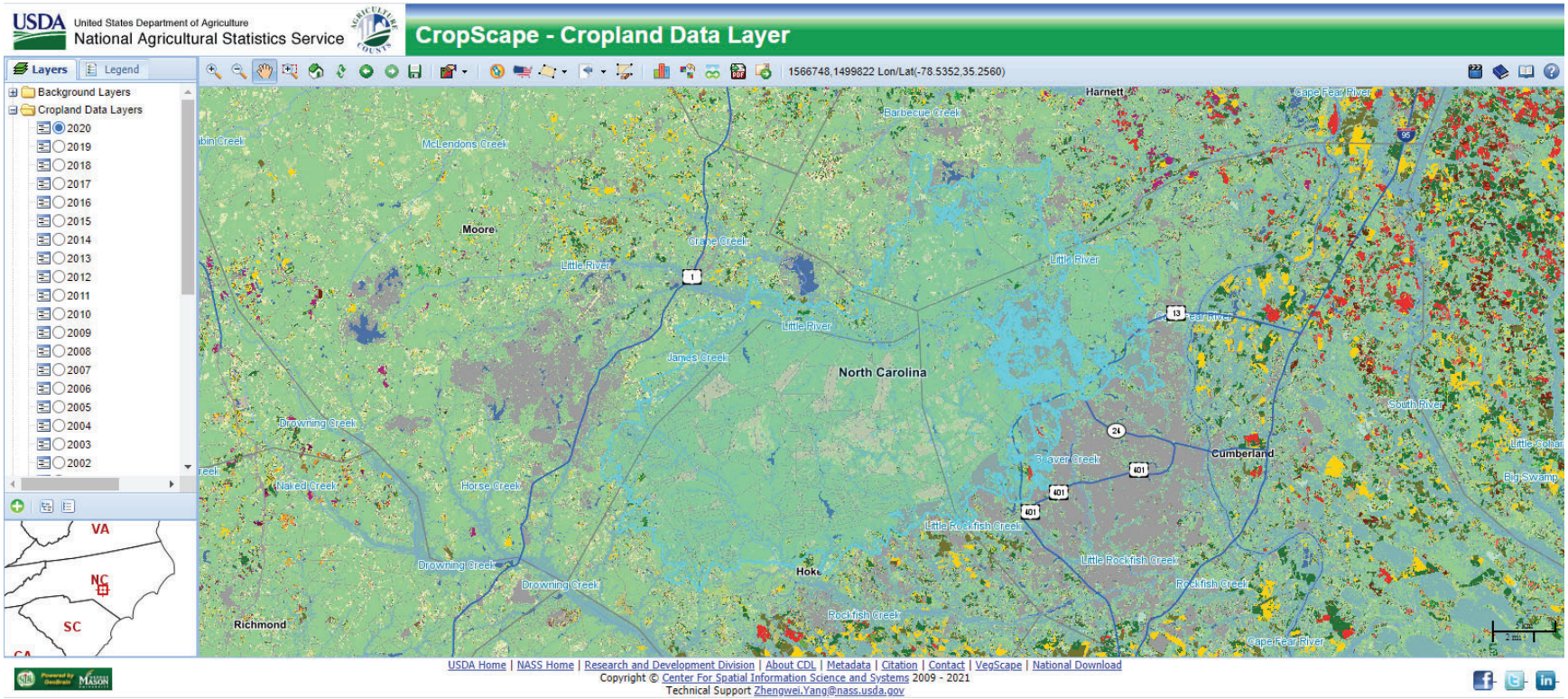

Figure L-7. Current Species Range for SFSB (accessed on 9/24/2021)

Current species range is indicated by blue polygons and the CDL for corn is indicated in yellow.

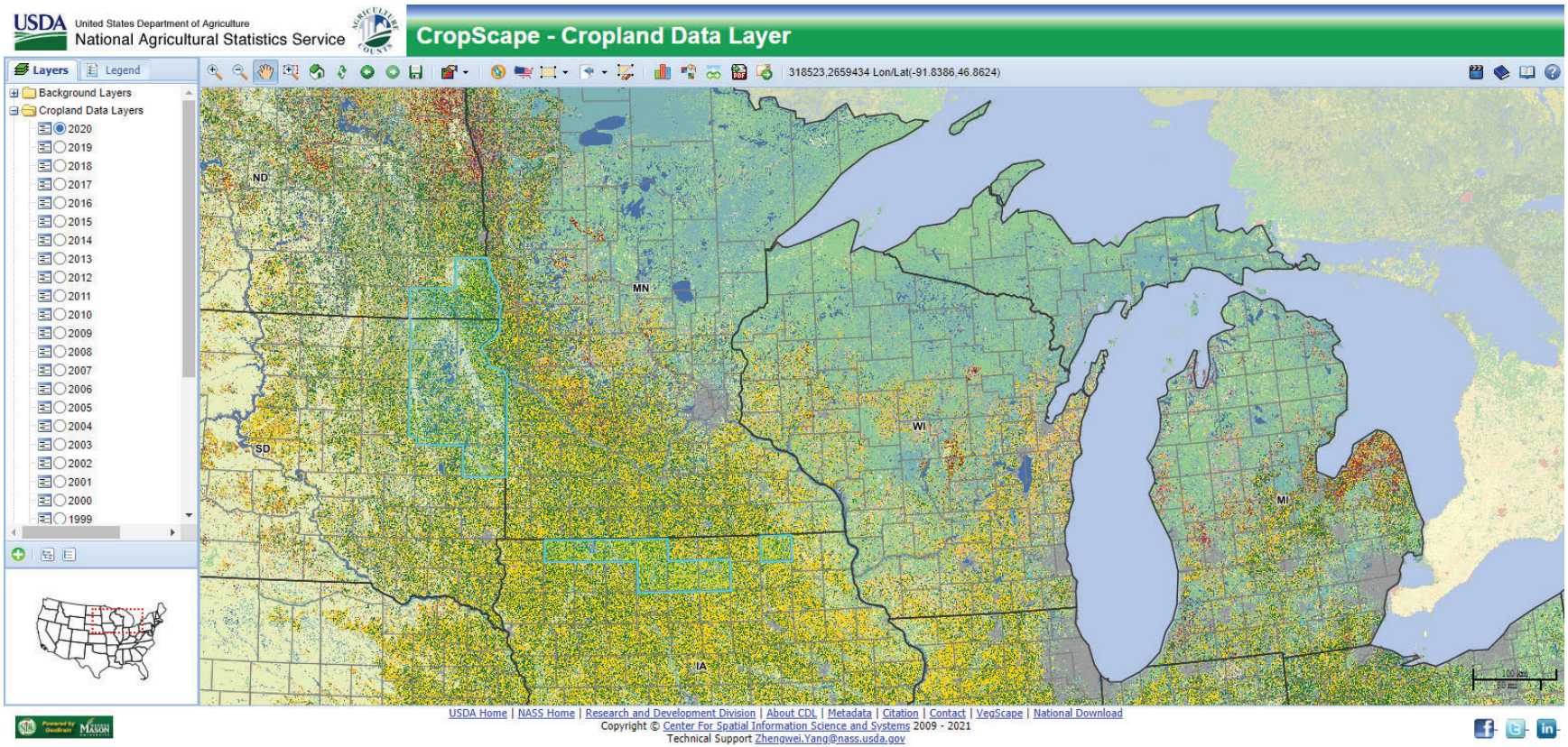

Figure L-8. Current National-Level Species Range for Poweshiek Skipperling (accessed 9/27/2021)

Historical and current species ranges are indicated by blue polygons and the CDL for corn is indicated in yellow. Species is no longer present in IA, ND, and SD (USFWS, 2018).

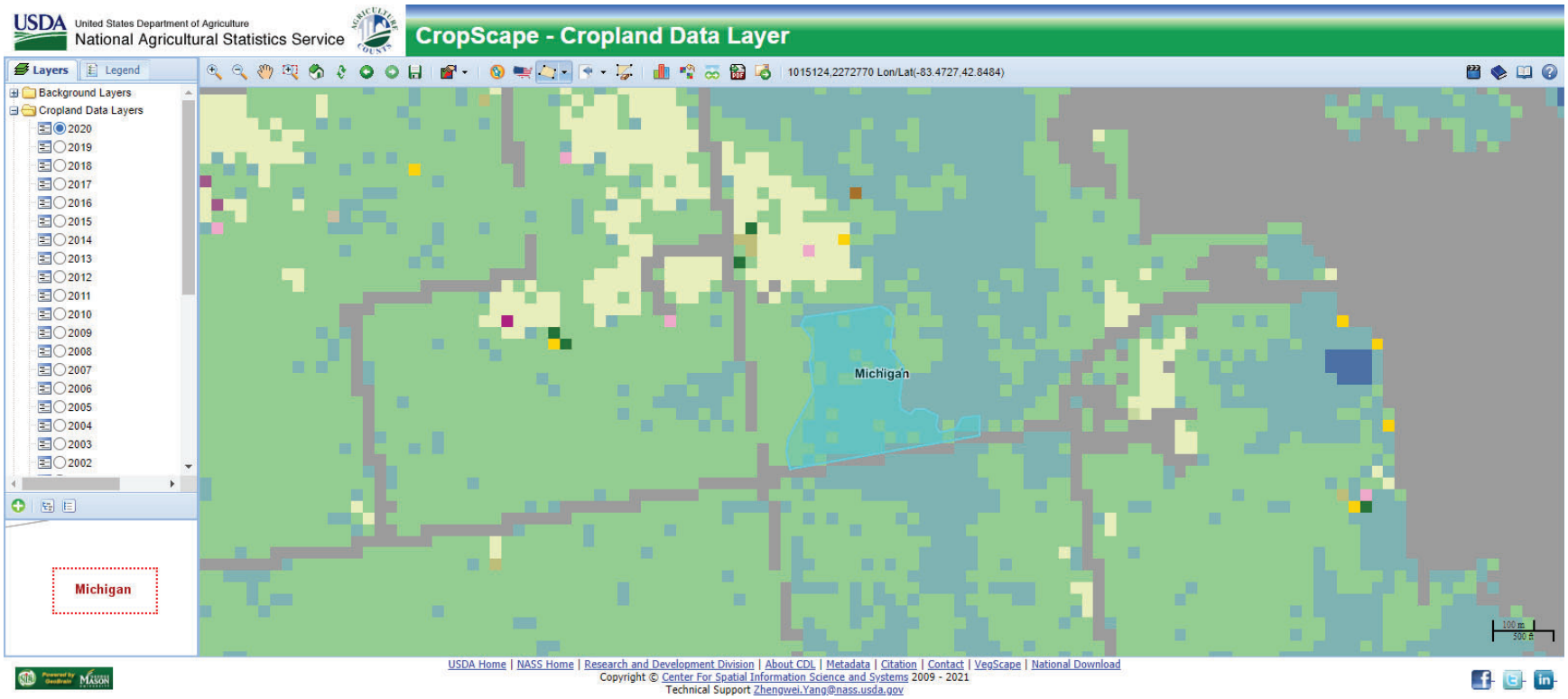

Figure L-9. Michigan Unit 1 Critical Habitat for Poweshiek Skipperling (accessed 9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

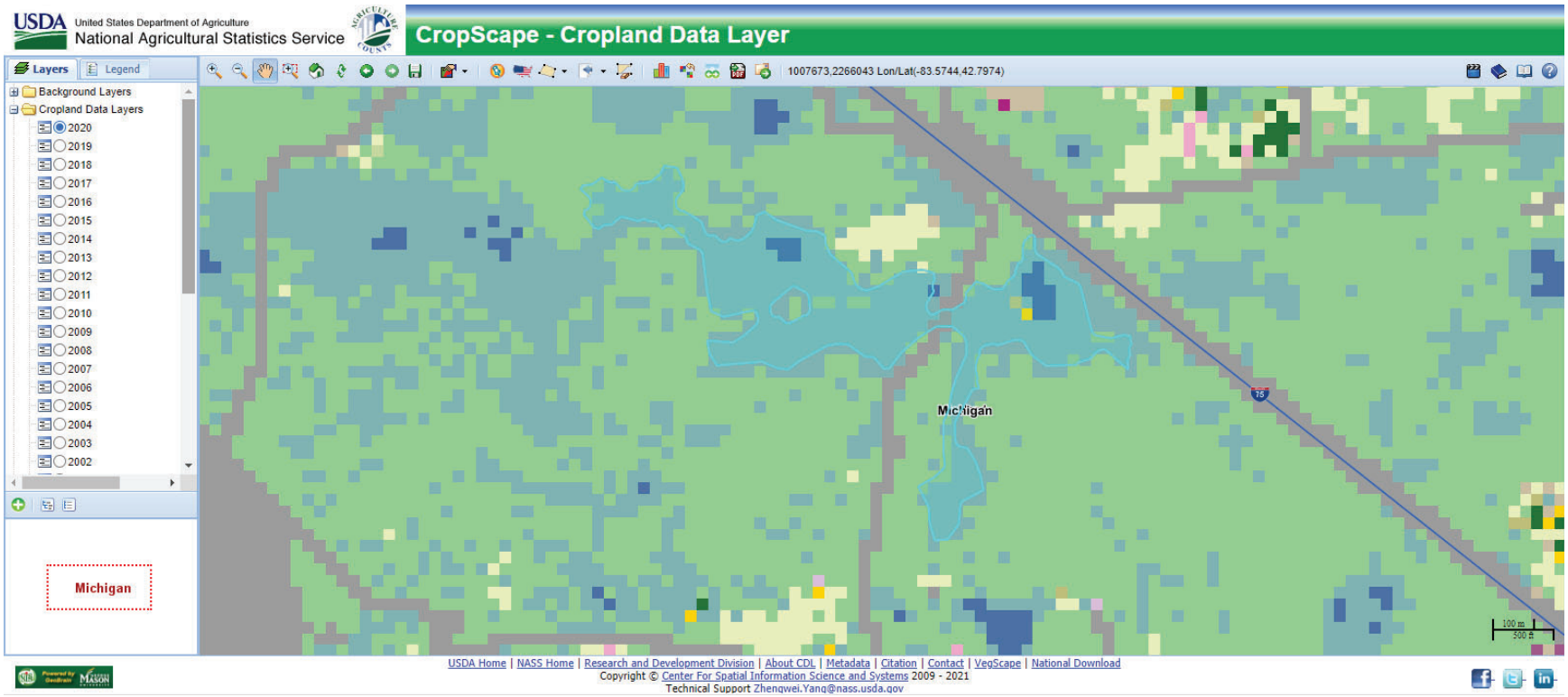

Figure L-10. Michigan Unit 5 Critical Habitat for Poweshiek Skipperling (accessed 9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

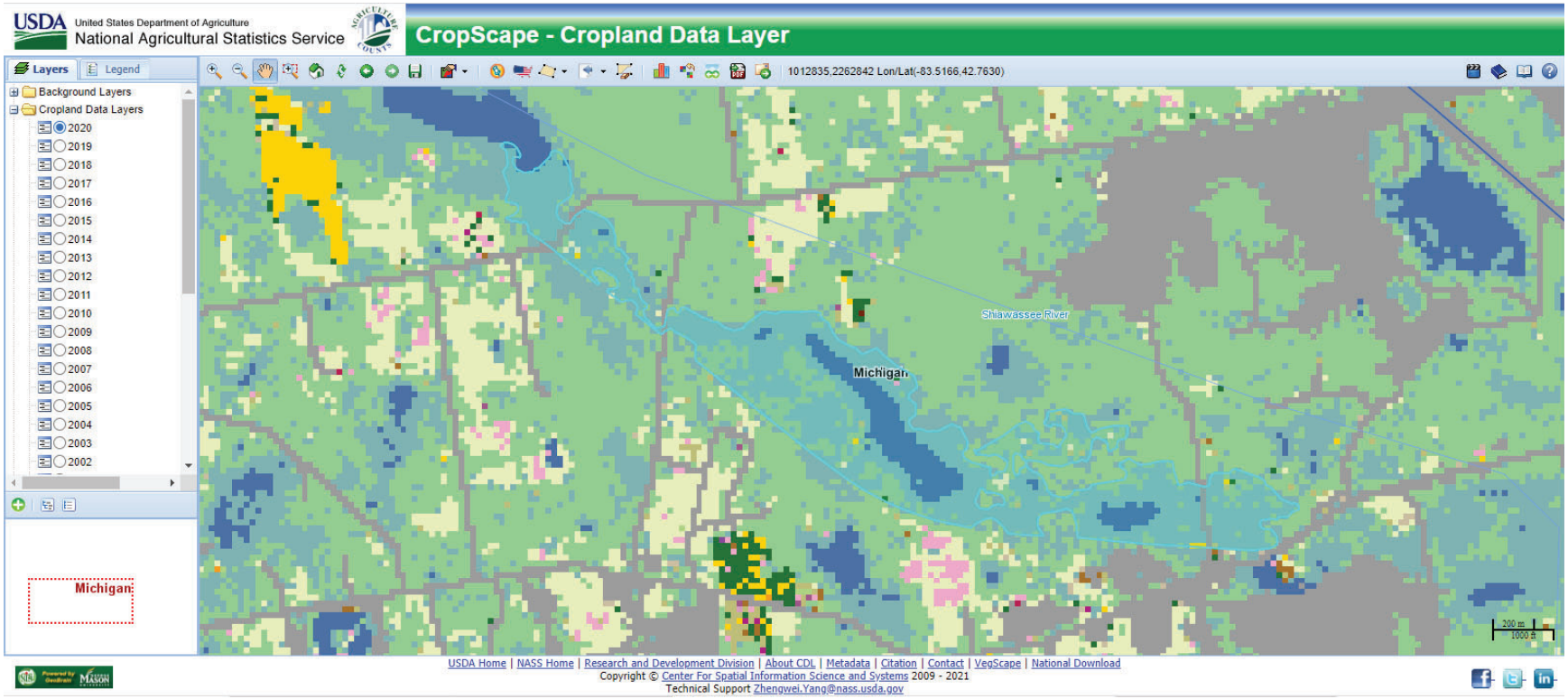

Figure L-11. Michigan Unit 3 Critical Habitat for Poweshiek Skipperling (accessed 9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

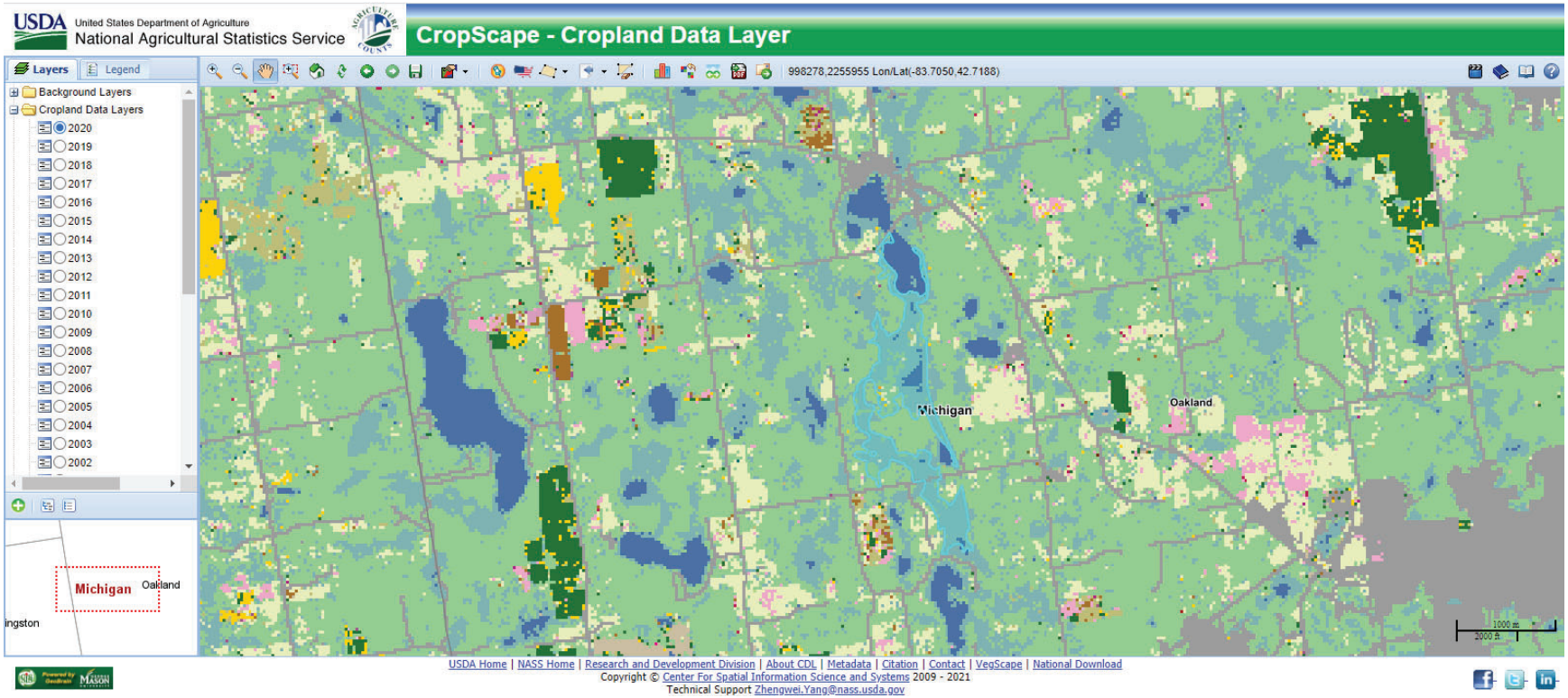

Figure L-12. Michigan Unit 4 Critical Habitat for Poweshiek Skipperling (accessed 9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

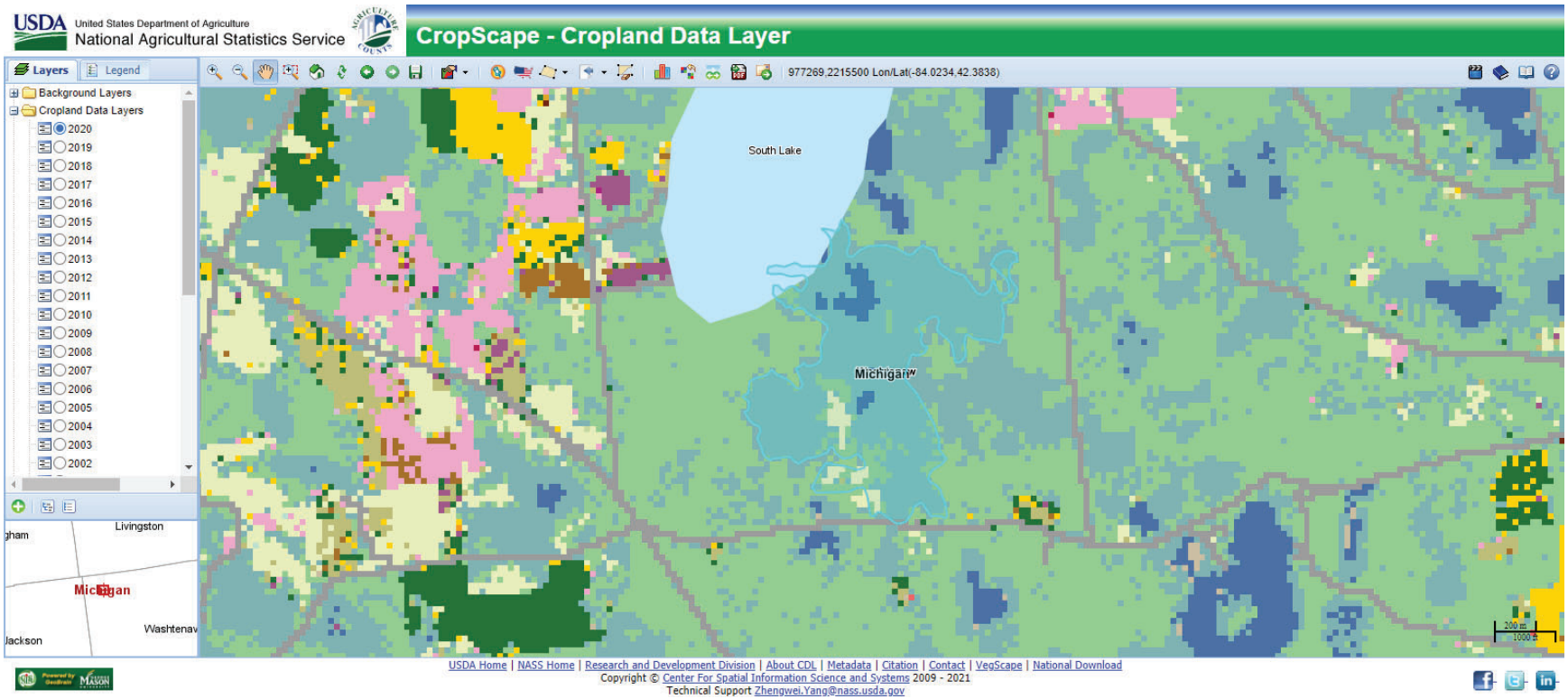

Figure L-13. Michigan Unit 6 Critical Habitat for Poweshiek Skipperling (9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

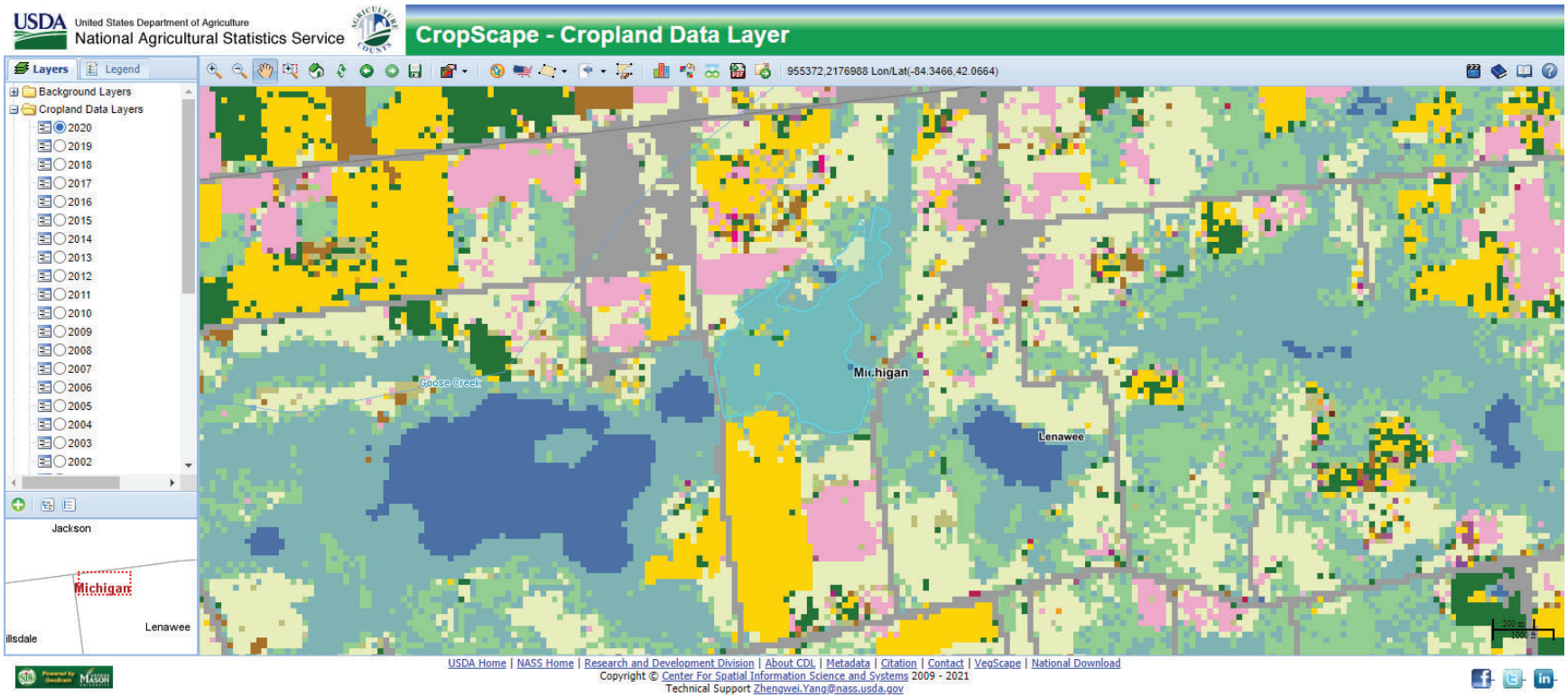

Figure L-14. Michigan Unit 7 Critical Habitat for Poweshiek Skipperling (accessed 9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

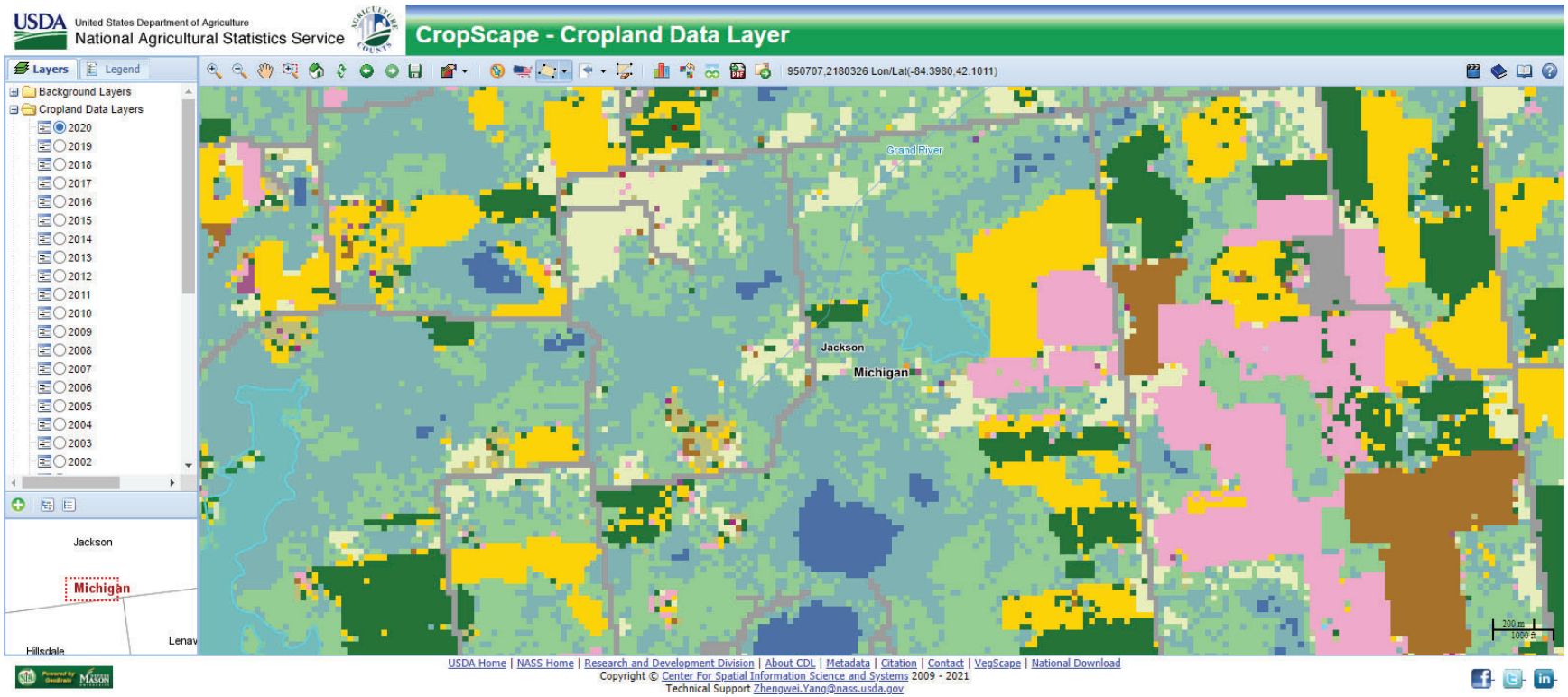

Figure L-15. Michigan Unit 9 Critical Habitat for Poweshiek Skipperling (accessed 9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

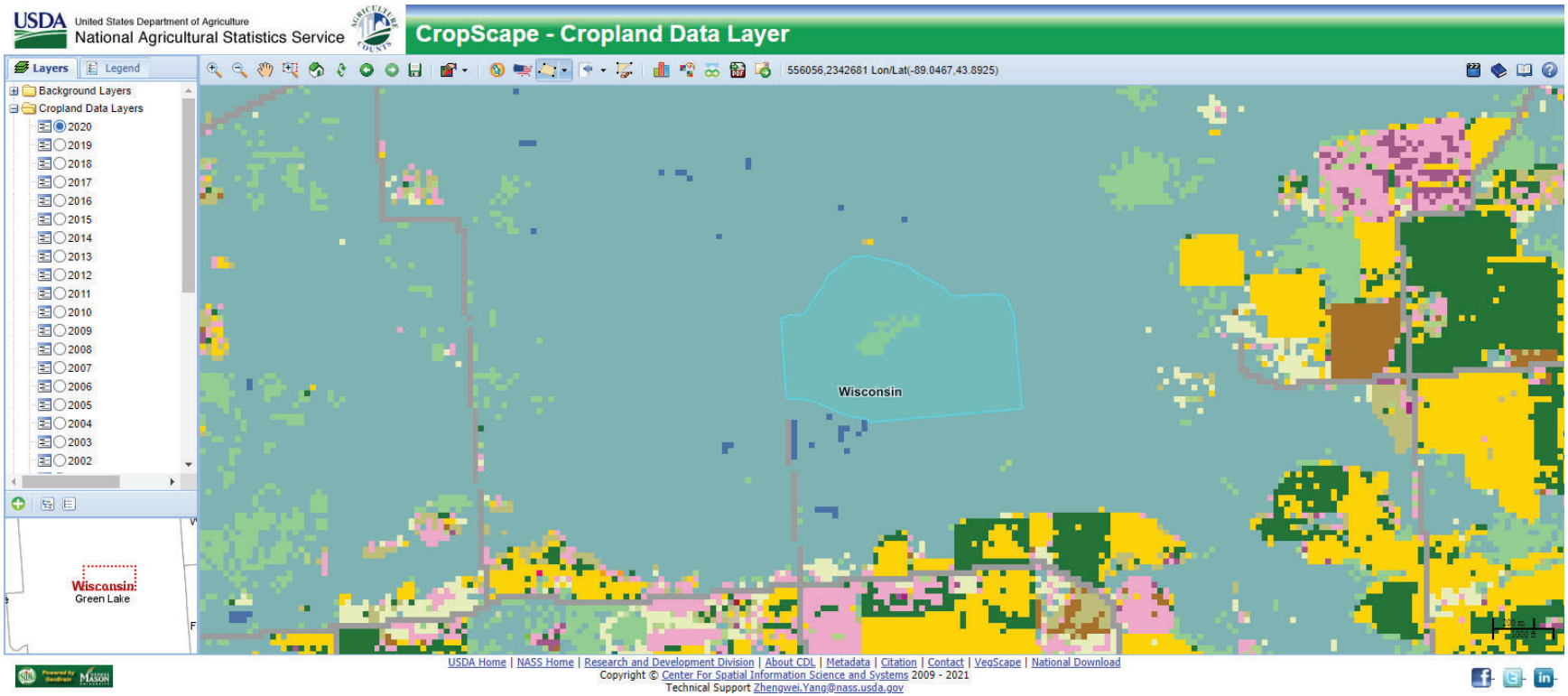

Figure L-16. Wisconsin Unit 2 Critical Habitat for Poweshiek Skipperling (accessed 9/23/2021)

The specified critical habitat range is indicated by blue polygons and the CDL for corn is indicated in yellow.

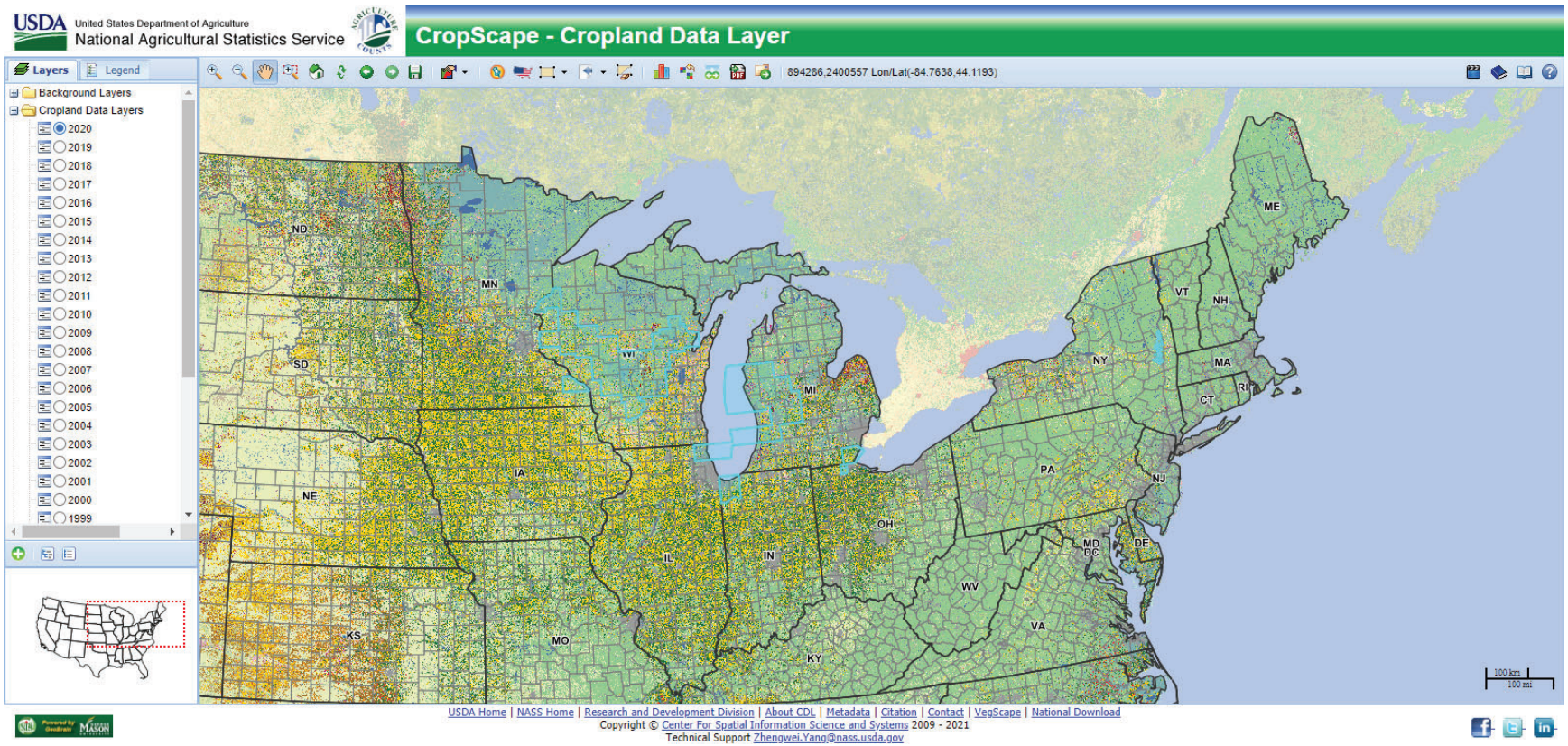

Figure L-17. Current Species Range for Karner Blue Butterfly (accessed 9/27/2021)

The current species range is indicated by blue polygons and the CDL for corn is indicated in yellow.

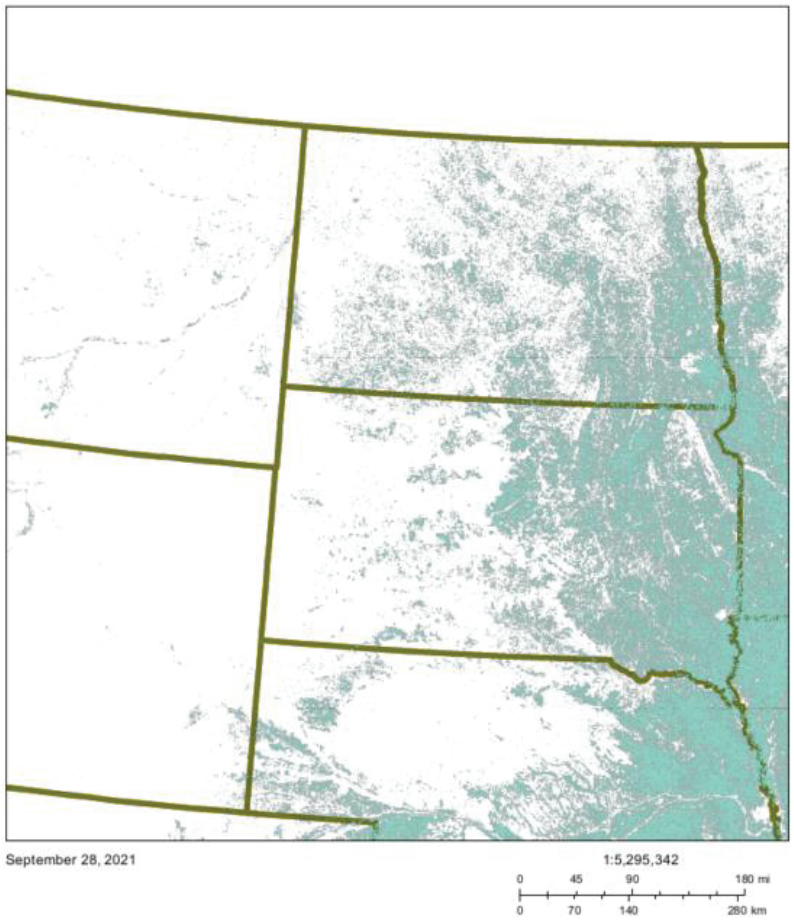

Figure L-18. Current Critical Habitat Range for Dakota Skipper (FESTF Gopher ;accessed 9/28/2021)

Range for DS critical habitat is indicated in purple and Use Data Layer (UDL) for Corn is indicated in green. Data Source: NatureServe and its natural heritage member programs.

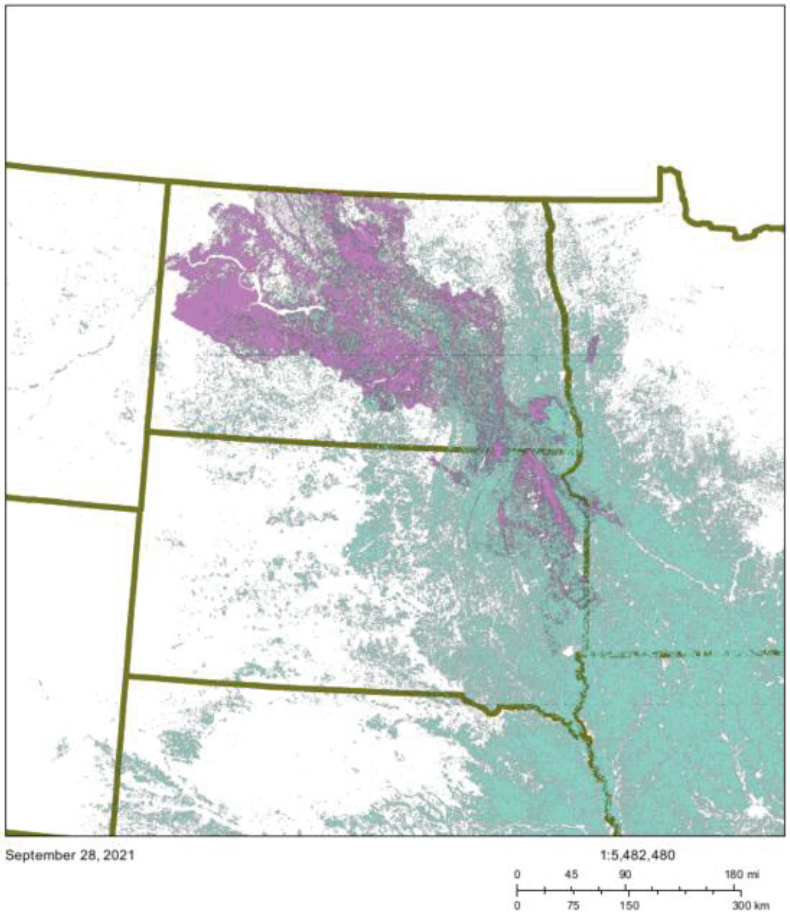

Figure L-19. Current Species Range for Dakota Skipper (FESTF Gopher; accessed 9/28/2021)

Species range for DS is indicated in purple and Use Data Layer (UDL) for Corn is indicated in green. Data Source: NatureServe and its natural heritage member programs.

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