Pioneer Hi-Bred International, Inc.
DP56113 SPTA Maintainer

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Petition for an Extension of the Determination of Nonregulated Status for MS44 Maintainer Line DP56113 for use in the Seed Production Technology for Africa (SPTA) Process

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OECD Unique Identifier: DP-Ø56113-9

Submission Date: February 10, 2020

DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

The undersigned submits this petition under 7 CFR §340.6 to request that the Administrator make a determination that the article, DP-Ø56113-9 maintainer maize (DP56113 SPTA Maintainer) not be regulated under 7 CFR §340.

Sally A. Catron — 02/10/20

Sally A. Catron — Date

Release of Information

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

Certification

The undersigned 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 relevant data and information known to the petitioner which are unfavorable to the petition.

02/10/20

Date

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Abbreviations, Acronyms, and Definitions

32138 SPT Maintainer	DP-32138-1 maize
5' UTR	5 prime untranslated region
ALS	Acetolactate synthase
amiRNA	Artificial microRNA
AM	Armyworm
AN	Anthracnose
AP	Aphid
APHIS	Animal and Plant Health Inspection Service
attB	Bacteriophage lambda integrase recombination site
ARC	Agricultural Research Council
BCW	Black cutworm
BNT	Below normal temperature
bp	Base pair
Bmt I	Restriction enzyme from *Bacillus megaterium* S2
BSR	Bacterial stalk rot
*Bst*E II	BstEII gene from *Bacillus stearothermophilus* ET
bu	bushel
bu/A	bushel per acre
BW	Buggy whipping
°C	Degrees Celsius
CA	California
CaMV 35S enhancer	35S enhancer region from the cauliflower mosaic virus genome
CD	Cold
CEW	Corn ear worm
CFIA	Canadian Food Inspection Agency
cm	centimeter
CMH	Cochran-Mantel-Haenszel
CMS	Cytoplasmic male sterility
colE1 *ori*	Origin of replication region from *Escherichia coli*
cos	Cohesive ends from lambda bacteriophage DNA
DD	Deer damage
DDG	Distillers dried grains
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DP-Ø56113-9	DP56113 SPTA maintainer maize
DR	Drought

Abbreviations, Acronyms, and Definitions (continued)

ds-red2	*Discosoma sp* red 2 gene
DsRed2	*Discosoma sp* red fluorescent protein
DTT	Dithiothreitol
ECB	European corn borer
EFSE	Early Food Safety Evaluation
EIA	United States Energy Information Administration
ELISA	Enzyme-linked immunosorbent assay
ER	Ear rot
ES	Eyespot
ETS	Excellence Through Stewardship
°F	Degrees Fahrenheit
F1	First filial generation
FAM	Fall armyworm
FB	Flea beetle
FDA	Food and Drug Administration
FDR	False discovery rate
FR	Frost
ft	feet
g	gram
GE	Genetically engineered
GH	Grasshopper
GLMM	Generalized linear mixed model
GLS	Gray leaf spot
GRAS	Generally recognized as safe
GW	Goss's bacterial wilt
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	Hawaii
HL	Hail
HLS	Holcus leaf spot
HPPD	4-Hydroxyphenylpyruvate dioxygenase
HRP	Horseradish peroxidase
HS	Heat stress
IA	Iowa
IL	Illinois
in	inches
JP	Japanese beetle
KALRO	Kenya Agricultural & Livestock Research Organization

Abbreviations, Acronyms, and Definitions (continued)

kDa	kilodalton
kg	kilogram
lb	Pound
LDS	Lithium dodecyl sulfate
LH	Leaf hopper
LLOQ	Lower limit of quantification
In2-1	Terminator region from the *Zea mays In2-1* gene
*lox*P	loxp recombination site
Ltp2	Barley (*Hordeum vulgare*) aleurone-specific lipid transfer protein gene
m^2	meter squared
mg	milligram
ml	milliliter
mM	milli molar
MN	Minnesota
mo-cre	maize-optimized (*mo*) versions of exon 1 and exon 2 of the *cre* recombinase gene from *Escherichia coli*
µg	microgram
MES	2-(N-morpholino) ethanesulfonic acid
MPI	Maintenance and non-target pesticide injury
MS44	MS44 protein
MS45	MS45 protein
NaOH	Sodium hydroxide
N/A	Not applicable
NC	Nitrocellulose
ND	Nutrient deficiency
NE	Nebraska
NIL	Near isoline
ng	nanogram
NGS	Next generation sequencing
NLS	Northern leaf spot
nm	nanometer
non-GE	non-genetically engineered
nos	*Agrobacterium tumefaciens* Ti plasmid nopaline synthase gene
NPC	New Protein Consultation
OD	Optical density

Abbreviations, Acronyms, and Definitions (continued)

ODP2	Ovule development protein 2
OECD	Organization for Economic Co-operation and Development
ORF	Open reading frame
PA	Pennsylvania
PBST	Phosphate-buffered saline containing polysorbate 20
PCR	Polymerase chain reaction
PHB00	Pioneer proprietary inbred line
PH251F	Pioneer proprietary inbred line
PH1CJB	Pioneer proprietary inbred line
PH24E	Pioneer proprietary inbred line
PH3KP	Pioneer proprietary inbred line
PHCER	Pioneer proprietary inbred line
PHCJP	Pioneer proprietary inbred line
PHEJW	Pioneer proprietary inbred line
PHH5G	Pioneer proprietary inbred line
*pin*II	*Solanum tuberosum* (potato) proteinase inhibitor II gene
Pg47	Maize pollen-specific polygalacturonase gene
PR	Puerto Rico
p-value	Probability value
qPCR	Qualitative polymerase chain reaction
*rep*A	Replication operon region A from *Agrobacterium rhizogenes*
*rep*B	Replication operon region B from *Agrobacterium rhizogenes*
*rep*C	Replication operon region C from *Agrobacterium rhizogenes*
RFP	Red fluorescent protein
RFU	Relative fluorescence units
RSC	Common maize rust
RW	Adult root worm
SB	Sap beetle
SbS	Southern-by-Sequencing
Sca I	Restriction enzyme from *Streptomyces caespitosus*
SCP	Soil crusting
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Standard error
SG	Slug
SM	Spider mite

Abbreviations, Acronyms, and Definitions (continued)

SMT	Smut
spc	Spectinomycin resistance gene from bacteria
SPT	Seed Production Technology
SPTA	Seed Production Technology for Africa
SR	Stalk rot
SS	Sun scald
SSC	Saline sodium citrate
SW	Stewart's wilt
T0	Transformation generation
T1	First selfed generation
T2	Second selfed generation
T3	Third selfed generation
T4	Fourth selfed generation
T-DNA	Transfer deoxyribonucleic acid
TH	Thrip
TZ	Tetrazolium chloride
UB	Unknown blight
*ubi*ZM1	*Zea mays* ubiquitin gene 1
USDA	United States Department of Agriculture
V	Volt
WA	Washington
WD	Wind damage
WG	White grub
WL	Waterlogging
WUS	Wuschel 2 protein
zm-aa1	*Zea mays* alpha amylase gene
ZM-AA1	*Zea mays* alpha amylase protein
zm-bt1	*Zea mays* amyloplast membrane protein *Brittle-1 gene*
zm-ms44	*Zea mays ms44* gene
zm-ms45	*Zea mays ms45* gene
zm-odp2	*Zea mays* ovule development protein 2 gene
zm-rab17	*Zea mays RAB-17* gene
zm-wus2	*Zea mays Wuschel2* gene

Summary

Pioneer Hi-Bred International, Inc. (Pioneer), member of Corteva Agriscience group of companies, is submitting an Extension Petition for Determination of Nonregulated Status for MS44 maintainer line maize event DP-Ø56113-9, hereafter referred to as DP56113 SPTA maintainer. Pioneer requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that the DP56113 SPTA maintainer, DP56113 SPTA maintainer progeny, and any crosses of DP56113 SPTA maintainer with other nonregulated maize no longer be considered regulated articles under 7 CFR §340, and that APHIS consider this petition as an extension to petition 08-338-01p under 7 CFR §340.6(e). Maize DP-32138-1 (hereafter referred to as 32138 SPT maintainer) was presented in 08-338-01p and received a determination of non-regulated status on May 24, 2011. DP56113 SPTA maintainer exhibits the same phenotype as 32138 SPT maintainer: male fertility restoration (silencing of the MS44 gene in DP56113 SPTA maintainer, complementing the MS45 gene in 32138 maize) to promote pollen fertility and alpha-amylase (ZM-AA1) protein expression to deplete pollen starch supply. Both events also contain expression of the DsRed2 protein as a visible sorting marker.

Pioneer has previously developed a process called 'Seed Production Technology' (SPT) that facilitates in-house production of male-sterile maize lines that are used as female inbred parents for subsequent hybrid seed production. This process is also utilized by DP56113 SPTA maintainer, now called 'Seed Production Technology for Africa' (SPTA). Pioneer, in collaboration with the Bill and Melinda Gates Foundation, the International Maize and Wheat Improvement Center (CIMMYT), the Kenya Agricultural & Livestock Research Organization (KALRO), and the Agricultural Research Council (ARC) has developed SPTA, which provides a reliable and predictable method to produce high quality hybrid maize seed for small and medium size seed companies in Africa.

Hybrid maize seed production requires crossing two inbred parent lines to produce hybrid seed sold to growers. The female inbred parent must be prevented from shedding pollen to avoid self-pollination that reduces hybrid seed quality and compromises hybrid yield potential. SPTA offers advantages over other approaches to controlling male-fertility in female inbred parents, such as detasseling (physical removal of the pollen-producing tassels) or cytoplasmic male-sterility (CMS). The SPTA process requires no detasseling, works across germplasm types, increases inbred seed yield, and produces F1 hybrid plants that are fertile. The female inbred parent seed produced using this technology do not contain the DP56113 SPTA maintainer insertion and consequently, hybrid seed produced using these male-sterile female inbred parents likewise do not contain the DP56113 SPTA maintainer insertion and hence are non-genetically engineered (non-GE) for SPTA.

DP56113 SPTA maintainer was created by *Agrobacterium*-mediated transformation of a genetically male sterile mutant maize line heterozygous for *Ms44* (*Ms44/-)* with plasmid PHP70533. The inserted transfer DNA (T-DNA) region from plasmid PHP70533 contains six cassettes, three of which are essential for the functioning of the SPTA system: *Ms44*, *zm-aa1*, and *DsRed2*. The remaining cassettes (*zm-wus2*, *zm-odp2, and mo-cre* gene cassettes) contain the *wus2* and *odp2* genes to enhance tissue regeneration after transformation, and the *cre* recombinase gene for facilitation of site specific recombination of the *lox*P sites during transformation and plant regeneration. Following T-DNA integration, recombination of *lox*P sites within the inserted T-DNA removes the *wus2*, *zm-odp2, and mo-cre* gene cassettes to result in a final insertion containing three cassettes.

The zm-*Ms44* gene is a dominant male-sterile mutant that can be used to produce male-sterile hybrids. Expression of an artificial micro RNA (amiRNA) targeting and silencing the zm-*Ms44* gene restores male fertility in a *Ms44* mutant background, enabling the production of maintainer lines (Fox et al., 2017). *Ms44* fertility is restored by silencing *Ms44* expression using amiRNA which restores pollen production. The same effect was achieved in 32138 SPT maintainer by replacing a male-sterile mutation (ms45) with a wild-type allele (MS45).

The *zm-aa1* gene cassette in DP56113 SPTA Maintainer is identical to the antecedent 32138 SPT maintainer and contains a truncated maize α-amylase (*zm-aa1*) gene that encodes the ZM-AA1 protein. The *zm-aa1* gene is native to maize and the coding region in the DP56113 SPTA maintainer is preceded by the transit peptide from the maize *Brittle-1* (*zm-bt1*) gene that targets the ZM-AA1 protein to the amyloplast. The ZM-AA1 protein prevents accumulation of starch in the nascent pollen grain, thus preventing the pollen from developing and germinating normally.

The *DsRed2* gene cassette in DP56113 SPTA Maintainer is identical to the antecedent 32138 SPT maintainer and contains a modified red fluorescent protein (*DsRed2*) gene from *Discosoma sp.* in which an internal *Bst*E II restriction site was removed from the original *DsRed2* gene without altering the amino acid sequence of the expressed protein. The tissue-specific expression of the DSRed2 protein in the aleurone layer of the maize seed produces a red coloration in seeds that contain the DNA insertion, allowing for differentiation during seed sorting.

Molecular characterization of DP56113 SPTA maintainer by Southern blot analysis and a Next Generation Sequencing (NGS) method known as Southern-by-Sequencing (SbS™ technology, hereafter referred to as SbS) confirmed that a single, intact PHP70533 DNA fragment was inserted into the genome. Segregation analysis of DP56113 SPTA maintainer confirmed the Mendelian inheritance of the *zm-aa1* and *Ds-Red2* genes.

The allergenicity assessment of translated ORFs identified an unidentified rice (*Oryza sativa*) amino acid sequence with an 88% identity homology over the α-amylase portion (excluding the

transit peptide) of the ZM-AA1 protein in DP56113 SPTA maintainer. The up-regulated alpha amylase is not new (Hirano *et al.*, 2016; Lombard *et al.*, 2014), it has cross-reactivity with other allergens already in pollen (maize and Poaceae in general) (Besler *et al.*, 2001; Lombard *et al.*, 2014), and for those that have allergens to maize pollen, the precautions against sensitivity and allergenic reactions would be unchanged. The ZM-AA1 protein is native to maize, maize is not a common allergenic food and the modification of DP56113 SPTA maintainer is not expected to alter the allergenic potential of maize as a food source.

The DP56113 SPTA maintainer is neither a commercial product on its own nor part of a commercial product; there is no intent for the DP56113 SPTA maintainer seed to be sold as commercial seed. The DP56113 SPTA maintainer is currently grown under USDA-APHIS permits on Pioneer-controlled parent seed production fields that are reproductively isolated from other maize fields. Rather than obtaining a USDA-APHIS permit each year for these DP56113 SPTA maintainer acres, Pioneer is requesting that USDA-APHIS deregulate the DP56113 SPTA maintainer to facilitate its use in the inbred seed production process, which is intended to be utilized to supply high quality inbred seed to small and medium size seed companies in Africa.

The data and information contained herein supports the conclusion that DP56113 SPTA maintainer is similar to the deregulated antecedent organism, 32138 SPT maintainer. Therefore, Pioneer requests that APHIS grant the request for a determination of extension of nonregulated status for DP56113 SPTA maintainer, DP56113 SPTA maintainer progeny, and any crosses of DP56113 SPTA maintainer with other nonregulated maize.

No known information is available which would be unfavorable to this petition.

I. Rationale for the Development of DP56113 SPTA Maintainer

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

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has responsibility, under the Plant Protection Act of 2000, to prevent the introduction or dissemination of plant pests into or within the United States. 7 CFR §340 regulates introduction of organisms altered or produced through genetic engineering (GE) which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR §340.6 provide that an applicant may petition APHIS to evaluate submitted data on the GE crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

In addition, §340.6(e) of the regulations provides APHIS with the ability to extend a determination of nonregulated status to other regulated articles upon determination that these regulated articles do not pose a potential for plant pest risk based on the similarity to one or more antecedent organisms that have been determined to be nonregulated. An evaluation of similarity between DP56613 SPTA maintainer and its antecedent organism (32138 SPT maintainer maize) is presented in accordance with §340.6(e).

Pioneer is submitting data for genetically engineered DP56113 SPTA maintainer and requests a determination from USDA-APHIS that event DP56113 SPTA maintainer, its progeny, and any crosses with other nonregulated maize no longer be considered regulated articles under 7 CFR §340.

I-B. Rationale for the Development of DP56113 SPTA Maintainer

This petition serves as a request for extension of the “Petition for the Determination of Nonregulated Status for Maize 32138 SPT maintainer used in the Pioneer Seed Production Technology (SPT) Process”. The 32138 SPT maintainer received a determination of nonregulated status on May 24, 2011. DP56113 SPTA maintainer demonstrates the same phenotypes (pollen fertility restoration, alpha-amylase depletion of pollen starch, and visible red marker) as 32138 SPT maintainer. The rationale for development of DP56113 SPTA maintainer is the same as that presented for 32138 SPT maintainer: to enable the production of male sterile inbred lines for use in seed production.

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

DP56113 SPTA maintainer has been extensively field tested in the United States as authorized by the USDA-APHIS permits and notifications (Appendix 1).

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

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

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

The United States is a major global exporter of maize at approximately 38% of the total trade market (USDA-FAS, 2019) The largest maize United States export markets in 2018-2019 were Mexico, Japan, Colombia, South Korea, and Peru (NCGA, 2018a; U.S. Grains Council, 2019). United States exports accounted for 15.4% of the maize produced in 2018 and those exports were shipped to more than 70 countries (NCGA, 2018b; U.S. Grains Council, 2019).

A significant portion of maize cultivated in the United States is GE. In 2018, 92% of maize grown in the United States was planted to GE hybrids.

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

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

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

There are several means of dry milling maize grain, but by far the most widely used process begins with soaking the kernel in water to remove the pericarp and germ, followed by drying the remaining grain fraction before additional processing. Products from the dry milling process

include flour, meal, germ meal, oil, beverage and fuel ethanol, distillers dried grain/solubles (DDG), flaking grits, hominy feed, and grits. Maize grain may also be cooked in alkali and finely ground to produce what is known as *masa*, which is used for tortillas and snack chips (OECD, 2002)

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

I-D.2. Feed Use of Maize

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

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

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

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

I-D.3. Fuel Use of Maize

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

I-D.4. Food Use of Maize

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

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

I-E. Comparison of DP56113 SPTA Maintainer to Antecedent Organism 32138 SPT Maintainer

An evaluation of similarity between DP56613 SPTA maintainer and its antecedent organism (32138 SPT maintainer maize) is presented in accordance with §340.6(e), and as described in the USDA-APHIS BRS Request to Extend Nonregulated Status from a Previous Determination: Extension Guidance for Developers.

DP56113 SPTA maintainer is intended for use in producing high quality inbred lines as the male fertility restorer line in the SPTA program. In both DP56113 SPTA maintainer and 32138 SPT maintainer, the male fertility restorer line is produced using *Agrobacterium*-mediated transformation using a vector containing the *zm-aa1*, *Ds-Red2*, and a mechanism for restoring pollen fertility (amiRNA silencing cassette for maize dominant male sterile *zm-Ms44* gene in DP56113 SPTA maintainer; the mutation of native gene *zm-Ms45* in 32138 SPT maintainer to compliment the male sterile *zm-ms45* mutation). The *zm-aa1* gene encodes the ZM-AA1 protein which is an alpha-amylase that is expressed in developing pollen.

DP56113 SPTA maintainer and the antecedent organism 32138 SPT maintainer use identical mechanisms of action for the seed color marker (DsRed2), and pollen infertility (ZM-AA1).

I-E.1. Functional Equivalency of DP56113 SPTA Maintainer to 32138 SPT Maintainer

I-E.1.a. Mechanism of Action

DP56113 SPTA maintainer uses the same mechanisms of action for restoration of male fertility when compared to 32138 SPT maintainer.

The expressed MS45 protein restores fertility in GE 32138 SPT maintainer by enabling pollen production. However, the *Ms45* gene in 32138 SPT maintainer is hemizygous (*Ms45/-*) and as a result, only one half of the pollen produced contains the *Ms45* gene. That half of the pollen also contains the linked *zm-aa1* gene, so the expressed α-amylase enzyme degrades starch, rendering the GE *(Ms45/zm-aa1/DsRed2(Alt1))* pollen infertile. The remaining half of the pollen is non-GE for SPT, fertile, and carries the endogenous recessive *ms45* gene. Therefore, when 32138 SPT maintainer is used as a pollinator to propagate the seed of male-sterile female inbred lines that are non-GE for SPT, the derived progeny retains the male-sterility genotype (*ms45/ms45*) and do not contain the 32138 SPT insertion and are therefore non-GE for SPT.

Similarly, the expressed *MS44* amiRNA restores fertility in GE DP56113 SPTA maintainer by enabling pollen production, and is used in the same manner as 32138 SPT maintainer to produce male-sterile female inbred lines. The construct in DP56113 SPTA maintainer (containing Ms44 amiRNA, ZM-AA1, and DsRed2 cassettes) was transformed into a heterozygous *Ms44* male-sterile inbred line to produce a *Ms44* homozygous maintainer line. Pollen from DP56113 SPTA sheds

50% GE, unfertile *Ms44* pollen (with the linked alpha-amylase and DsRed genes) and 50% fertile, non-GE *Ms44* pollen. Like 32138 SPT maintainer, pollen containing the DP56113 SPTA maintainer construct are unable to germinate due to depletion of starch by expression of α-amylase (ZM-AA1 protein). Pollen that does not contain the DP56113 SPTA maintainer construct are able to fertilize *Ms44* mutant plants. The resulting *Ms44* inbred seeds do not contain the linked alpha-amylase or DsRed2 color marker, are non-GE, with respect to the DP56113 SPTA maintainer construct, and are able to be used for hybrid seed production. (Fox et al, 2017)

DP56113 SPTA maintainer and 32138 SPT maintainer lines also both utilize the expression of the DsRed2 protein in kernels containing the Ms44 and Ms45 wild type traits, respectively. Both maize lines use this visual marker to screen for GE kernels during parent seed production. Kernels containing the red visual marker are removed from the seed production process, leaving behind male sterile parent seed to be used in hybrid seed production.

Based on this comparison, the mechanisms of action used to achieve male sterility and visual marker kernel sorting are similar between DP56113 SPTA maintainer and 32138 SPT maintainer.

I-E.1.b. Crop Considerations

APHIS has previously made a determination of nonregulated status for restoration of pollen fertility, pollen starch depletion via expression of alpha-amylase, and visual marker kernel sorting for maize via the deregulation of 32138 SPT maintainer.

Tables 1 and 2 provide side-by-side comparisons of the DP56113 SPTA maintainer and 32138 SPT maintainer characteristics and genetic elements.

Table 1. Comparison of DP56113 SPTA Maintainer and Antecedent Organism

Characteristic	**32138 SPT Maintainer**	**DP56113 SPTA Maintainer**
Recipient Organism	*Zea Mays*	*Zea Mays*
Phenotype	Fertility restoration, 50% sterile pollen, color marker kernels	Fertility restoration, 50% sterile pollen, color marker kernels
Mechanism of Action	Fertility restoration via Ms45; Infertile pollen via starch depletion; Red color marker in GE kernels	Fertility restoration via Ms44 amiRNA; Infertile pollen via starch depletion; Red color marker in GE kernels
Gene Products	Ms45, α-amylase, DsRed2	*Zm-Ms44* amiRNA, α-amylase, DsRed2
Transformation Method	*Agrobacterium*-mediated	*Agrobacterium*-mediated
Vector	PHP24597	PHP70533

Table 2. Comparison of DP56113 SPTA Maintainer and Antecedent Organism Genetic Elements

	Characteristic	32138 SPT Maintainer	DP56113 SPTA Maintainer
Pollen infertility cassette	Gene/Donor	*zm-aa1* *Zea mays*	*zm-aa1* *Zea mays*
	Promoter/Donor	*Pg47* *Zea mays*	*Pg47* *Zea mays*
	Terminator/Donor	*In2-1* *Zea mays*	*In2-1* *Zea mays*
Color sorting cassette	Gene/Donor	*DsRed2* *Discosoma sp.*	*DsRed2* *Discosoma sp.*
	Promoter/Donor	*Ltp2* Barley	*Ltp2* Barley
	Terminator/Donor	*pin*II *Solanum tuberosum*	*pin*II *Solanum tuberosum*
Sterility restoration cassette	Gene/Donor	*ms45* *Zea mays*	*Ms44 ami-RNA* *Zea mays*
	Promoter/Donor	5126 *Zea mays*	*zm-Ms44* *Zea mays*
	Terminator/Donor	N/A	*zm-Ms44* *Zea mays*

II. The Biology of Maize

II-A. Maize as a Crop

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

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

The subsequent breeding of DP56113 SPTA maintainer proceeded as indicated in Figure 2 to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial maize lines.

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

A Pioneer proprietary line, PHH5G was used as the recipient line to produce DP56113 SPTA maintainer. Line PHH5G was chosen because it is receptive to transformation and is also an elite line (*i.e.*, Pioneer proprietary line used for commercial products).

III. Method of Development of DP56113 SPTA Maintainer

III-A. Description of Transformation, Selection, and Breeding Method for DP56113 SPTA Maintainer

DP56113 SPTA maintainer was created by *Agrobacterium*-mediated transformation with plasmid PHP70533 (Figure 3; Table 4). The inserted T-DNA region from plasmid PHP70533 (Figure 4; Table 5) contains six cassettes. Following T-DNA integration, recombination of *lox*P sites within the inserted T-DNA removed three cassettes to result in a final insertion containing three cassettes (Figure 5). Creation of transformation events and resultant plants from PHP70533 occurred in Johnston, Iowa, United States.

Pioneer proprietary inbred line PHH5G was transformed with plasmid PHP70533 to produce DP56113 SPTA maintainer. Immature maize embryos were harvested from a surface-sterilized ear of PHH5G maize approximately 8-11 days after pollination and inoculated with *Agrobacterium tumefaciens* strain LBA4404 containing plasmids PHP10523 and PHP70533 (Zhao et al., 2001). *Agrobacterium tumefaciens* strain LBA4404 is a disarmed strain that does not contain tumor-inducing factors; however, with the inclusion of plasmid PHP10523, the strain contains factors (*i.e.*, the *vir* genes) that enable the transfer of the T-DNA region to the inoculated host plant. After three to six days of embryo and *Agrobacterium* co-cultivation on solid culture medium without selection, the embryos were transferred to a medium with glufosinate herbicide selection and containing the antibiotic carbenicillin to kill residual *Agrobacterium*. Prior to plant regeneration, transformed callus was subjected to a brief desiccation step to activate the *zm-rab17* promoter (Vilardell et al., 1990) and express the Cre protein. The Cre protein excised the DNA sequences between the two *lox*P sites in the PHP70533 T-DNA, comprising the *zm-wus2*, *zm-odp2*, and *mo-cre* gene cassettes, to yield the intended insertion (Figure 5). Callus was then transferred to germination medium and incubated to initiate shoot and root development. Once shoots and roots were established, healthy plants were selected, and PCR was used to confirm the presence of the PHP70533 T-DNA insert. Plants that were regenerated from transformation and tissue culture (designated T0 plants) were selected for further characterization and advancement through the breeding process. (Figure 1)

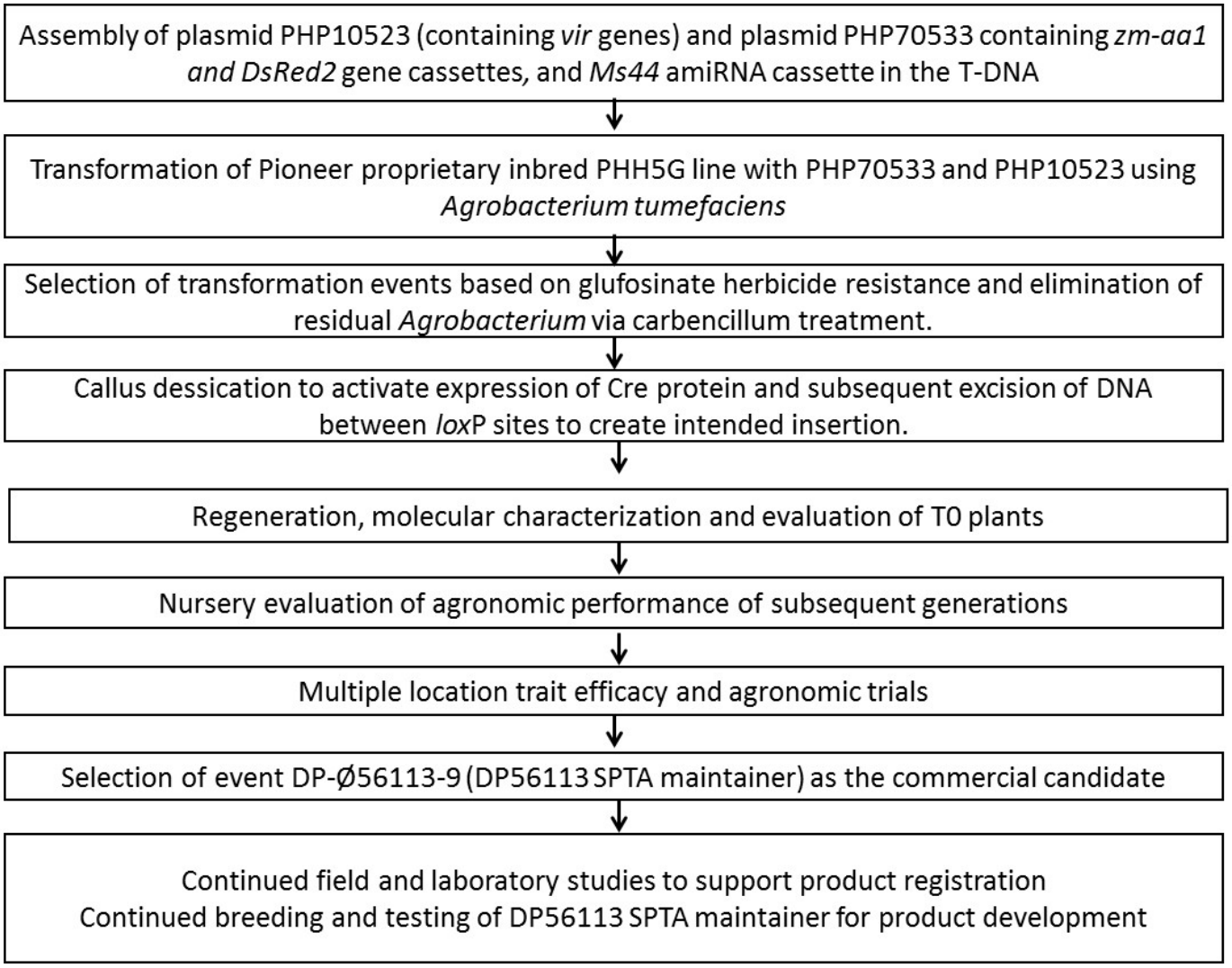


Figure 1. Schematic Diagram of the Development of DP56113 SPTA Maintainer

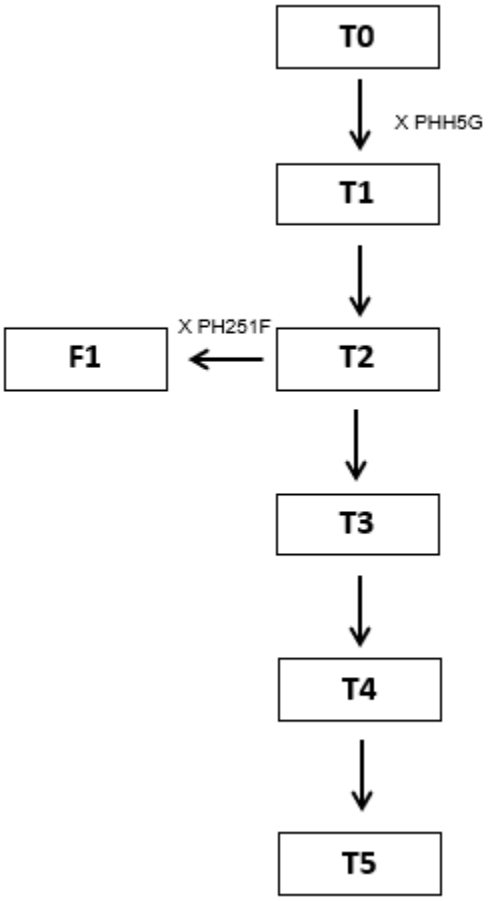


Figure 2. Breeding Diagram for DP56113 SPTA Maintainer

The breeding steps to produce the generations used for characterization, assessment, and the development of commercial lines are shown schematically in Figure 2 and are listed in Table 3. Pioneer proprietary maize inbred PHH5G was used for transformation to produce DP56113 SPTA maintainer. Pioneer proprietary maize inbred line PH251F was used in crossing steps.

Table 3. Generations and Comparators Used for Analysis of DP56113 SPTA Maintainer

Analysis	Seed Generation(s) Used	Comparators
Copy Numbers, Integrity, and Backbone by SbS	T1	PHH5G
Integrity and Stability by Southern Blot	T1, T2, T3, and T4	PHH5G
Agronomic and Expression Analysis	T4	PHH5G
Mendelian Inheritance	T2, T3, T4, T5, and F1	N/A

III-B. Selection of Comparators for DP56113 SPTA Maintainer

For the characterization of DP56113 SPTA maintainer, Pioneer proprietary maize inbred line PHH5G was used as an experimental control (Table 3). The control lines selected represent the genetics of the maize lines used to produce the DP56113 SPTA maintainer generations used in analysis (Figure 2).

IV. Donor Genes and Regulatory Sequences in DP56113 SPTA Maintainer

IV-A. DP56113 SPTA Maintainer DNA Used in Transformation

Maize (*Zea mays* L.) was transformed by *Agrobacterium*-mediated transformation with plasmid PHP70533 (Figure 3). The T-DNA region of this plasmid is represented schematically in Figure 4. Summaries of the genetic elements and their positions on plasmid PHP70533 and on the T-DNA are provided in Tables 4 and 5, respectively.

The T-DNA of plasmid PHP70533 contains five gene cassettes and an amiRNA cassette. The first cassette (*zm-wus2* gene cassette) contains the maize *Wuschel2* (*wus2*) gene (Mayer et al., 1998) encoding the WUS protein. The expressed WUS protein enhances tissue regeneration after transformation (Lowe et al., 2016). The WUS protein is 302 amino acids in length and has a molecular weight of approximately 31 kDa. Expression of the *wus2* gene is controlled by the promoter from the *Agrobacterium tumefaciens* Ti plasmid nopaline synthase (*nos*) gene (Depicker et al., 1982), in conjunction with the terminator region from the potato (*Solanum tuberosum*) proteinase inhibitor II (*pin*II) gene (An et al., 1989; Keil et al., 1986).

The second cassette (*zm-odp2* gene cassette) contains the maize ovule development protein 2 (*odp2*) gene (GenBank accession XM008676474) encoding the ODP2 protein. The expressed ODP2 protein enhances the regeneration of maize plants from tissue culture after transformation (U.S. Patent US8420893B2). The ODP2 protein is 710 amino acids in length and has a molecular weight of approximately 74 kDa. Expression of the *odp2* gene is controlled by the promoter region from the maize ubiquitin gene 1 (*ubi*ZM1) including the 5' untranslated region (5' UTR) and intron (Christensen et al., 1992). The terminator for the *odp2* gene is a second copy of the *pin*II terminator.

The third cassette (*mo-cre* gene cassette) contains maize-optimized (*mo*) versions of exon 1 and exon 2 of the *cre* recombinase gene from *Escherichia coli* (Dale and Ow, 1990), separated by an intron region from the potato *LS1* (*st-LS1*) gene (Eckes et al., 1986). The expressed Cre protein facilitates site specific recombination of the *lox*P sites during transformation and plant regeneration. The Cre protein is 343 amino acids in length and has a molecular weight of approximately 39 kDa. Expression of the *mo-cre* gene is controlled by the promoter and 5'UTR regions from the maize RAB-17 (*zm-rab17*) gene (Vilardell et al., 1990), in conjunction with a third copy of the *pin*II terminator.

The fourth cassette (*zm-aa1* gene cassette) contains a truncated version of the maize α-amylase (*zm-aa1*) gene (Schnable et al., 2009) encoding the ZM-AA1 protein. The *zm-aa1* coding region is preceded by the sequence encoding the transit peptide from the maize amyloplast membrane

protein *Brittle-1* (*zm-bt1*) gene (Sullivan et al., 1991) that targets the ZM-AA1 protein to the amyloplast. The ZM-AA1 protein contributes to altered starch content (Chao and Scandalios, 1971) and prevents accumulation of starch in the nascent pollen grain, thus preventing the pollen from developing and germinating normally and rendering the pollen infertile. The complete translation product, including transit peptide, is 495 amino acids in length and has a molecular weight of approximately 54 kDa. The processed ZM-AA1 protein, with the transit peptide removed, is 420 amino acids in length and has a molecular weight of approximately 46 kDa. The processed ZM-AA1 protein differs from the native protein in that it lacks the 21 N-terminal amino acid residues found in the native protein, including the initial methionine residue. Expression of the transcript containing the *zm-bt1* transit peptide sequence and *zm-aa1* gene is controlled by the pollen-specific promoter from the maize polygalacturonase (*Pg47*) gene (Allen and Lonsdale, 1993), in conjunction with the terminator region from the maize *In2-1* gene (Hershey and Stoner, 1991).

The fifth cassette (*DsRed2* gene cassette) contains a modified version of the *Discosoma sp.* (coral anemone) red fluorescent protein (*DsRed2*) gene (Clontech, 2001), in which an internal *Bst*E II restriction site was removed from the original *DsRed2* gene without altering the amino acid sequence of the expressed protein. The tissue-specific expression of the DS-RED2 protein in the aleurone layer of the maize seed produces a red coloration in seeds that contain the DNA insertion, allowing for differentiation during seed sorting. The DSRed2 protein is 225 amino acids in length and has a molecular weight of approximately 26 kDa. Expression of the *DsRed2* gene is controlled by the 35S enhancer region from the cauliflower mosaic virus genome (CaMV 35S enhancer; Franck et al., 1980; Kay et al., 1987) and the promoter region from the barley (*Hordeum vulgare*) lipid transfer protein (*Ltp2*) gene (Kalla et al., 1994), which provide aleurone-specific transcription of the *DsRed2* gene. The terminator for the *DsRed2* gene is a fourth copy of the *pin*II terminator.

The sixth cassette (*zm-Ms44* amiRNA cassette) contains a sequence that expresses a transcript forming an artificial microRNA (amiRNA) precursor comprised of the *zm*-miRNA 5' precursor 396h, *zm-Ms44* amiRNA, *zm*-miRNA precursor 396h*, zm-Ms44* star sequence, and *zm*-miRNA 3' precursor 396h (U.S. Patent Application Publication No. 20090155909A1). Initial processing of the amiRNA precursor results in the folding of the transcript into a hairpin structure, pairing the *zm-Ms44* amiRNA and the *zm-Ms44* star sequence, which is complementary except for a single nucleotide mismatch. Further processing releases the *zm-Ms44* amiRNA that targets the *Ms44* gene for silencing. Expression of this transcript is controlled by the promoter and terminator regions from the maize *Ms44* gene (Fox et al., 2017).

The PHP70533 T-DNA contains two *lox*P (Dale and Ow, 1990) and six *attB* recombination sites (Cheo et al., 2004; Hartley et al., 2000; Katzen, 2007).

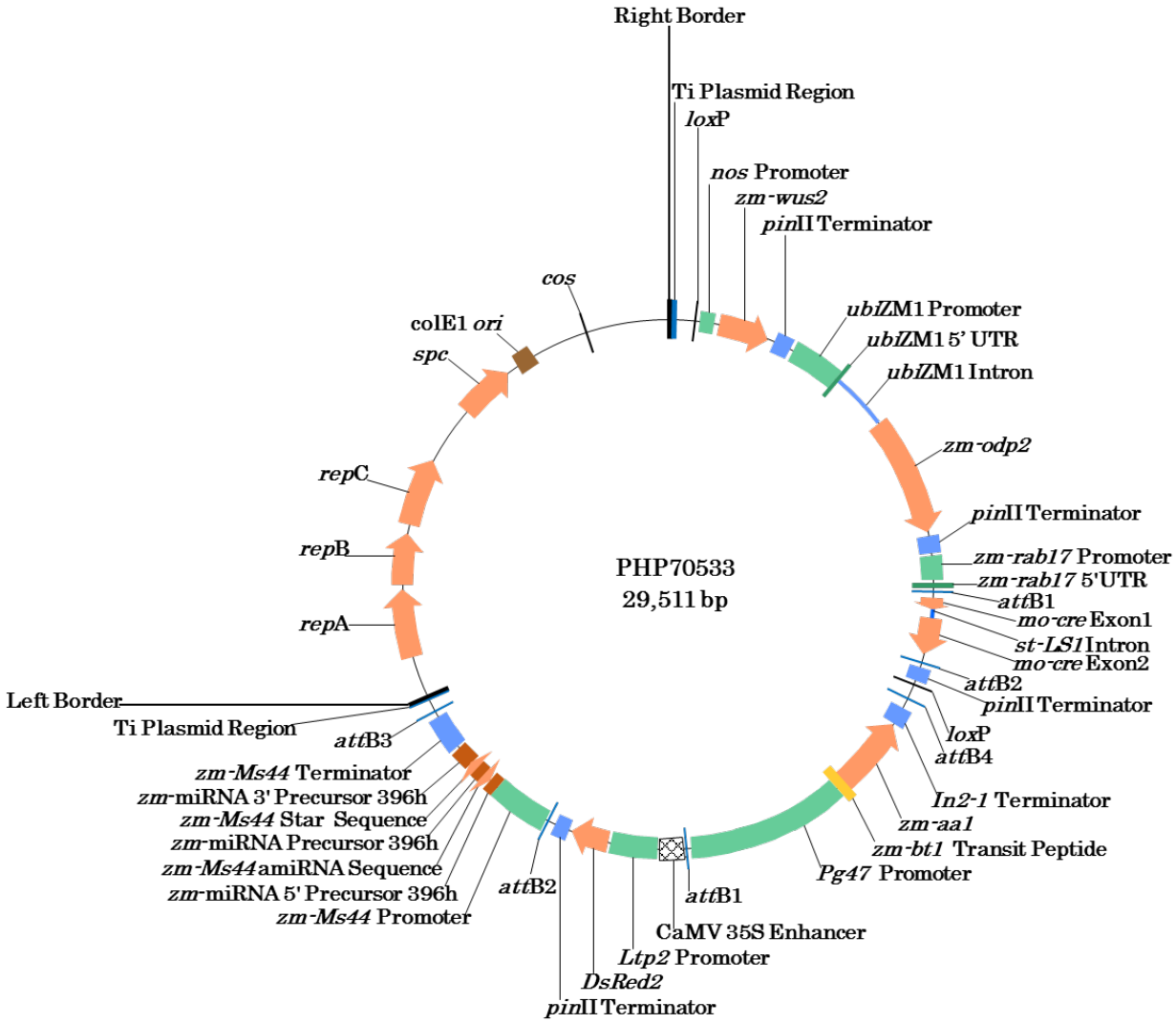


Figure 3. Schematic Diagram of Plasmid PHP70533

Schematic diagram of plasmid PHP70533 containing the *zm-aa1* and *DsRed2* gene cassettes and the *zm-Ms44* amiRNA cassette intended for incorporation into the maize genome, and the *zm-wus2*, *zm-odp2*, and *mo-cre* cassettes not intended for incorporation into the maize genome. The size of plasmid PHP70533 is 29,511 bp.

Table 4. Description of Genetic Elements in Plasmid PHP70533

Region	Location on Plasmid (bp to bp)	Genetic Element	Size (bp)	Description
T-DNA	1 – 20,081		20,081	See Table 2 for information on the elements in this region
Plasmid Construct	20,082 – 29,511	Includes Elements Below	9,430	DNA from various sources for plasmid construction and plasmid replication
	20,813 – 22,027	*rep*A	1,215	Replication operon region A from *Agrobacterium rhizogenes* (Nishiguchi et al., 1987)
	22,108 – 23,046	*rep*B	939	Replication operon region B from *Agrobacterium rhizogenes* (Nishiguchi et al., 1987)
	23,199 – 24,416	*rep*C	1,218	Replication operon region C from *Agrobacterium rhizogenes* (Nishiguchi et al., 1987)
	25,482 – 26,492	*spc*	1,011	Spectinomycin resistance gene from bacteria (Fling et al., 1985)
	26,578 – 26,947 (complementary)	colE1 *ori*	370	Origin of replication region from *Escherichia coli* (Tomizawa et al., 1977)
	28,041 – 28,054	*cos*	14	Cohesive ends from lambda bacteriophage DNA (Komari et al., 1996)

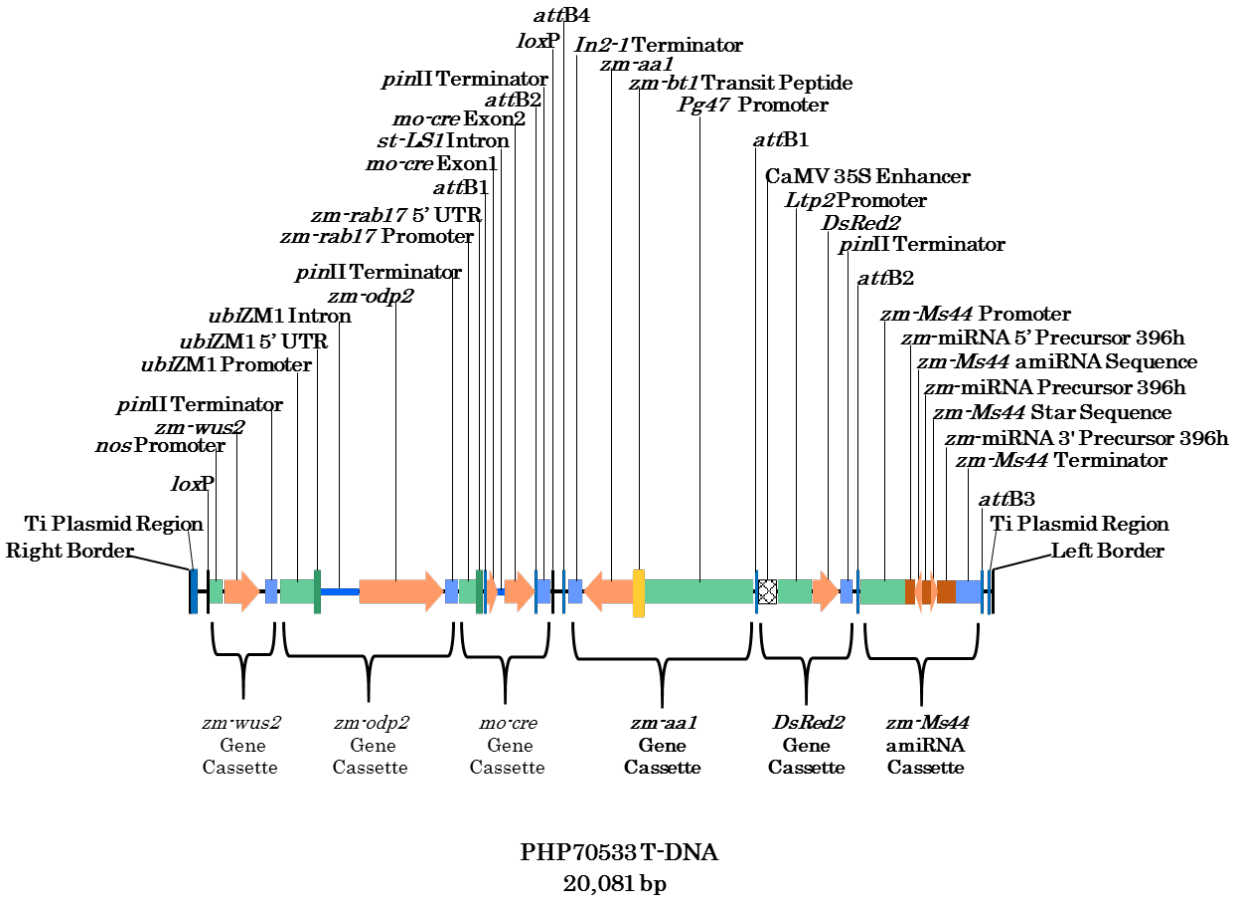


Figure 4. Schematic Diagram of the T-DNA Region from Plasmid PHP70533

Schematic diagram of the T-DNA region of plasmid PHP70533 indicating the *zm-aa1* and *DsRed2* gene cassettes and the *zm-Ms44* amiRNA cassette intended for incorporation into the maize genome, and the *zm-wus2*, *zm-odp2*, and *mo-cre* gene cassettes not intended for incorporation into the maize genome, defined as the fragment from bp 1 to bp 20,081. The size of the T-DNA is 20,081 bp.

Table 5. Description of Genetic Elements in the T-DNA Region from Plasmid PHP70533

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	1 – 25	Right Border (RB)	25	T-DNA Right Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)
	26 – 177	Ti Plasmid Region	152	Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)
	178 – 435	Intervening Sequence	258	DNA sequence used for cloning
	436 – 469	*lox*P	34	Bacteriophage P1 recombination site recognized by Cre recombinase (Dale and Ow, 1990)
	470 – 499	Intervening Sequence	30	DNA sequence used for cloning
zm-wus2 gene cassette	500 – 821	*nos* Promoter	322	Promoter region from the *Agrobacterium tumefaciens* Ti plasmid nopaline synthase gene (Depicker et al., 1982)
	822 – 874	Intervening Sequence	53	DNA sequence used for cloning
	875 – 1783	*zm-wus2*	909	*Wuschel2* gene from *Zea mays* (Lowe et al., 2016; Mayer et al., 1998)
	1,784 – 1,895	Intervening Sequence	112	DNA sequence used for cloning
	1,896 – 2,206	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	2,207 – 2,268	Intervening Sequence	62	DNA sequence used for cloning
zm-odp2 gene cassette	2,269 – 3,168	*ubi*ZM1 Promoter	900	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	3,169 – 3,251	*ubi*ZM1 5' UTR	83	5’ untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	3,252 – 4,264	*ubi*ZM1 Intron	1,013	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen et al., 1992)
	4,265 – 4,282	Intervening Sequence	18	DNA sequence used for cloning
	4,283 – 6,415	*zm-odp2*	2,133	Ovule development protein 2 gene from *Zea mays* (U.S. Patent US8420893B2; GenBank accession XM008676474)
	6,416 – 6,483	Intervening Sequence	68	DNA sequence used for cloning

Table 5. Description of Genetic Elements in the T-DNA Region from Plasmid PHP70533 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	6,484 – 6,794	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	6,795 – 6,832	Intervening Sequence	38	DNA sequence used for cloning
mo-cre gene cassette	6,833 – 7,340	*zm-rab17* Promoter	508	Promoter region from the *Zea mays* RAB-17 gene (Vilardell et al., 1990)
	7,341 – 7,433	*zm-rab17* 5' UTR	93	5' untranslated region from the *Zea mays* RAB-17 gene (Vilardell et al., 1990)
	7,434 – 7,473	Intervening Sequence	40	DNA sequence used for cloning
	7,474 – 7,497	*att*B1	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning system (Hartley et al., 2000; Katzen, 2007)
	7,498 – 7,522	Intervening Sequence	25	DNA sequence used for cloning
	7,523 – 7,766	*mo-cre* Exon1	244	Maize-optimized exon 1 of the *cre* recombinase gene from *Escherichia coli* (Dale and Ow, 1990)
	7,767 – 7,955	*st-LS1* Intron	189	Intron region from the *Solanum tuberosum* (potato) *LS1* gene (Eckes et al., 1986)
	7,956 – 8,743	*mo-cre* Exon2	788	Maize-optimized exon 2 of the *cre* recombinase gene from *Escherichia coli* (Dale and Ow, 1990)
	8,744 – 8,748	Intervening Sequence	5	DNA sequence used for cloning
	8,749 – 8,772	*att*B2	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning System (Hartley et al., 2000; Katzen, 2007)
	8,773 – 8,786	Intervening Sequence	14	DNA sequence used for cloning
	8,787 – 9,094	*pin*II Terminator[a]	308	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II (An et al., 1989; Keil et al., 1986)
	9,095 – 9,152	Intervening Sequence	58	DNA sequence used for cloning
	9,153 – 9,186	*lox*P	34	Bacteriophage P1 recombination site recognized by Cre recombinase (Dale and Ow, 1990)

[a] This copy of the *pin*II terminator is 3 bp shorter at the 5' end than the other *pin*II terminators in this vector.

Table 5. Description of Genetic Elements in the T-DNA Region from Plasmid PHP70533 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	9,187 – 9,439	Intervening Sequence	253	DNA sequence used for cloning
	9,440 – 9,460	*att*B4	21	Bacteriophage lambda integrase recombination site (Cheo et al., 2004)
	9,461 – 9,618	Intervening Sequence	158	DNA sequence used for cloning
zm-aa1 gene cassette	9,619 – 9,961 (complementary)	*In2-1* Terminator	343	Terminator region from the *Zea mays In2-1* gene (Hershey and Stoner, 1991)
	9,962 – 9,968	Intervening Sequence	7	DNA sequence used for cloning
	9,969 – 11,302 (complementary)	*zm-aa1*	1,334	Truncated version of the α-amylase gene from *Zea mays* including 3' untranslated region (UTR) (Schnable et al., 2009) as described below: 3' UTR at bp 9,969-10,039 (71 bp long) Coding sequence at bp 10,040-11,302 (1,263 bp long)
	11,303 – 11,529 (complementary)	*zm-bt1* Transit Peptide	227	Amyloplast-targeting transit peptide of the *Brittle-1* gene from *Zea mays* including 5' untranslated region (UTR) (Sullivan et al., 1991) as described below: Coding sequence at bp 11,303-11,527 (225 bp long) 5' UTR at bp 11,528-11,529 (2 bp long)
	11,530 – 14,265 (complementary)	*Pg47* Promoter	2,736	Promoter region from the *Zea mays* pollen-specific polygalacturonase gene (Allen and Lonsdale, 1993)
	14,266 – 14,332	Intervening Sequence	67	DNA sequence used for cloning
	14,333 – 14,356	*att*B1	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning system (Hartley et al., 2000; Katzen, 2007)
	14,357 – 14,391	Intervening Sequence	35	DNA sequence used for cloning
DsRed2 gene cassette	14,392 – 14,866	CaMV 35S Enhancer	475	35S enhancer region from the cauliflower mosaic virus genome (Franck et al., 1980; Kay et al., 1987)
	14,867 – 14,899	Intervening Sequence	33	DNA sequence used for cloning
	14,900 – 15,745	*Ltp2* Promoter	846	Promoter region from the *Hordeum vulgare* (barley) aleurone-specific lipid transfer protein gene (Kalla et al., 1994)

Table 5. Description of Genetic Elements in the T-DNA Region from Plasmid PHP70533 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
DsRed2 gene cassette (continued)	15,746 – 15,789	Intervening Sequence	44	DNA sequence used for cloning
	15,790 – 16,467	*DsRed2*	678	Modified version of the red fluorescent protein *DsRed2* gene from *Discosoma sp.* (coral anemone) (Clontech, 2001) with an internal *Bst*E II restriction site removed
	16,468 – 16,508	Intervening Sequence	41	DNA sequence used for cloning
	16,509 – 16,819	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	16,820 – 16,940	Intervening Sequence	121	DNA sequence used for cloning
	16,941 – 16,964	attB2	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning System (Hartley et al., 2000; Katzen, 2007)
	16,965 – 17,005	Intervening Sequence	41	DNA sequence used for cloning
zm-Ms44 amiRNA cassette	17,006 – 18,226	*zm-Ms44* Promoter	1,221	Promoter region from the *Zea mays Ms44* gene (Fox et al., 2017)
	18,227 – 18,229	Intervening Sequence	3	DNA sequence used for cloning
	18,230 – 18,311	*zm*-miRNA 5' Precursor 396h	82	5' precursor sequence of the microRNA backbone 396h from *Zea mays* (U.S. Patent US20090155909A1) (McGonigle, 2012)
	18,312 – 18,332 (complementary)	*zm-Ms44* amiRNA Sequence	21	Artificial microRNA (amiRNA) sequence complementary to the *Ms44* gene from *Zea mays* (U.S. Patent Application Publication No. 20090155909A1) (Fox et al., 2017)
	18,333 – 18,400	*zm*-miRNA Precursor 396h	68	Precursor sequence of the microRNA backbone 396h from *Zea mays* (U.S. Patent US20090155909A1) (McGonigle, 2012)
	18,401 – 18,421	*zm-Ms44* Star Sequence	21	Artificial star sequence complementary to the *zm-Ms44* amiRNA sequence except for one mismatched nucleotide (U.S. Patent Application Publication No. 20090155909A1) (Fox et al., 2017;)
	18,422 – 18,862	*zm*-miRNA 3' Precursor 396h	441	3' precursor sequence of the microRNA backbone 396h from *Zea mays* (U.S. Patent Application Publication No. 20090155909A1) (McGonigle, 2012)
	18,863 – 18,880	Intervening Sequence	18	DNA sequence used for cloning
	18,881 – 19,655	*zm-Ms44* Terminator	775	Terminator region from the *Zea mays Ms44* gene (Fox et al., 2017)

Table 5. Description of Genetic Elements in the T-DNA Region from Plasmid PHP70533 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	19,656 – 19,753	Intervening Sequence	98	DNA sequence used for cloning
	19,754 – 19,774	*att*B3	21	Bacteriophage lambda integrase recombination site (Cheo et al., 2004)
	19,775 – 19,999	Intervening Sequence	225	DNA sequence used for cloning
	20,000– 20,056	Ti Plasmid Region	57	Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)
	20,057 – 20,081	Left Border (LB)	25	T-DNA Left Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al., 1996)

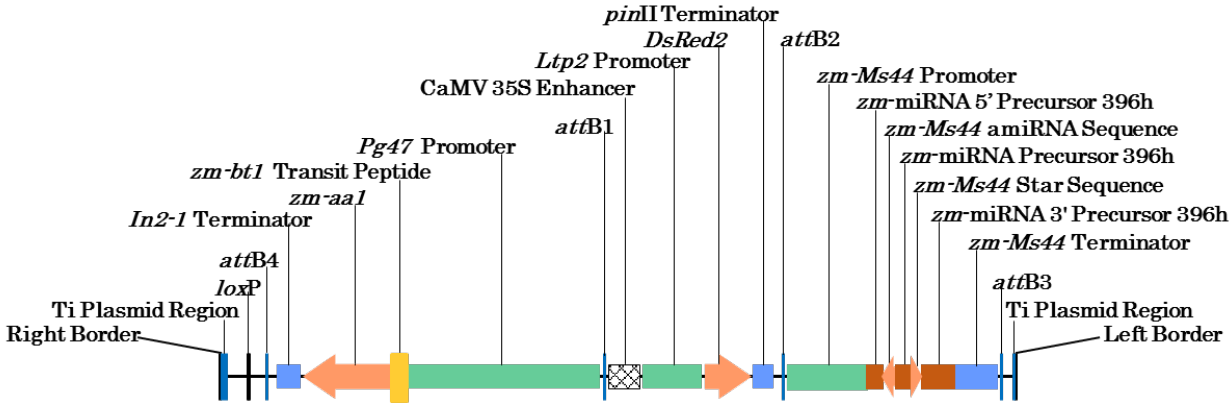


Figure 5. Schematic Map of Intended DP56113 SPTA Maintainer Insertion

Schematic map of the DNA insertion intended to be present in the maize genome following T-DNA integration and Cre-mediated recombination of the *lox*P sites. The size of the intended insertion after recombination is 11,364 bp. The *zm-aa1* and *DsRed2* gene cassettes and the *zm-Ms44* amiRNA cassette remain in the insertion in DP56113 SPTA maintainer.

V. Genetic Characterization of DP56113 SPTA Maintainer

V-A. DP56113 SPTA Maintainer Molecular Analysis Overview

Molecular characterization of GE events determines the insertion copy number, integrity of the insertion, and absence of plasmid DNA unintended for integration. The inserted DNA is also evaluated over several generations of plants to confirm its stable Mendelian inheritance. DP56113 SPTA maintainer plants were characterized by a Next Generation Sequencing (NGS) method known as Southern-by-Sequencing (SbS™ technology, hereafter referred to as SbS) method to determine the number of insertions within the plant genome, insertion integrity, and to confirm the absence of plasmid backbone sequences. Southern blot analysis was performed to confirm stable genetic inheritance of the inserted cassettes.

Based on the SbS analysis described below, it was determined that a single, intact insertion of the intended PHP70533 T-DNA, was inserted into the genome of DP56113 SPTA maintainer and that no sequences from the backbone of plasmid PHP70533 were present. In addition, Southern blot analysis across five breeding generations confirmed the stable genetic inheritance of the DNA insertion in DP56113 SPTA maintainer.

V-B. Southern by Sequencing (SbS) Analysis for Copy Number, Integrity, and Confirmation of the Absence of Vector Backbone Sequence in DP56113 SPTA Maintainer

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

The SbS technique (Figure 6) utilizes capture probes homologous to the transformation plasmid to isolate genomic DNA that hybridizes to the probe sequences (Zastrow-Hayes et al., 2015). Captured DNA is then sequenced using a NGS procedure and the results are analyzed using bioinformatics tools. During the analysis, junction reads are identified as those sequence reads where part of the read shows exact homology to the plasmid DNA sequence while the rest of the read does not match the contiguous plasmid. Junctions may occur between inserted DNA and genomic DNA, or between insertions of two plasmid-derived DNA sequences that are not contiguous in the transformation plasmid. Multiple sequence reads are generated for each junction and are compiled into a consensus sequence for the junction. By compiling a large

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

A schematic diagram of the SbS process is presented in Figure 6.

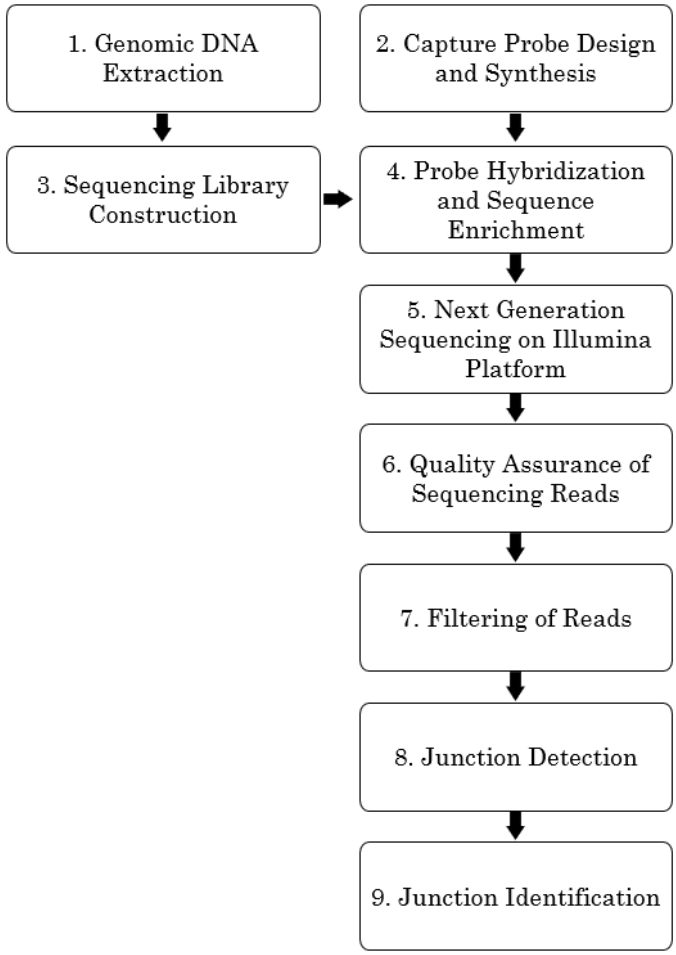


Figure 6. Southern by Sequencing (SbS) Process Flow Diagram

The T1 generation of DP56113 SPTA maintainer was analyzed by SbS, using full-coverage probes comprising the entire sequence of the PHP70533 transformation plasmid, to determine the insertion copy number and intactness and to confirm the absence of plasmid backbone sequences. SbS was also performed on control maize, and on a positive control sample to confirm that the assay could reliably detect plasmid fragments spiked into control maize genomic DNA. Based on the results obtained in this study, a schematic diagram of the DP56113 SPTA maintainer insertion was developed and is provided in Figure 7.

Several genetic elements in the PHP70533 T-DNA are derived from maize and thus the homologous elements in the PHH5G maize genome are be captured by the full-coverage probes used in the SbS analysis. These endogenous elements (*ubi*ZM1 promoter, 5' UTR, and intron, *zm-rab17* promoter and 5' UTR, *Pg47* and *zm-Ms44* promoters, *In2-1* and *zm-Ms44* terminators, *zm-wus2*, *zm-odp2*, *zm-bt1* transit peptide, *zm-aa1*, and the *zm-Ms44* amiRNA precursor; (Table 6, Figures 3-5) have sequencing reads in the SbS results due to the homologous elements in the PHH5G maize genome. However, if no junctions are detected, these sequencing reads only indicate the presence of the endogenous elements in their normal context of the maize genome and are not from inserted DNA.

SbS analysis results for the control maize and the positive control sample are presented in Figures 8 and 9, respectively. Results for the DP56113 SPTA maintainer T1 plant are presented in Figure 10. Coverage of sequencing reads is shown aligned to the PHP70533 plasmid map, the PHP70533 T-DNA map, and the intended DNA insertion following recombination of the *lox*P sites in the T-DNA.

Sequencing reads were detected in the PHH5G control maize (Figure 8); however, coverage above background level (35x) was obtained only for the genetic elements derived from the maize genome. These sequence reads were due to capture and sequencing of these genetic elements in their normal context within the PHH5G control maize genome. Variation in coverage of the endogenous elements is due to sequence variations between the PHH5G control maize and the maize varieties from which the genetic elements in PHP70533 were derived. No junctions were detected between plasmid sequences and the maize genome, indicating that there are no PHP70533 plasmid DNA insertions in the control maize, and the sequence reads were solely due to the endogenous genetic elements present in the PHH5G control maize genome.

SbS analysis of the positive control sample resulted in sequence coverage across the entire length of the plasmid (Figure 9), indicating that the SbS assay utilizing the full-coverage probe library is sensitive enough to detect PHP70533 sequences at a concentration equivalent to one copy of PHP70533 per copy of the maize genome. No junctions were detected between plasmid and genomic sequences, indicating that the sequence reads were due to either the spiked-in plasmid or the endogenous maize genetic elements that were detected in the control maize.

SbS analysis of the T1 generation of DP56113 SPTA maintainer resulted in two unique genome-plasmid junctions (Figure 10). The 5' junction started with bp 23 within the Right Border of the intended insertion derived from the PHP70533 T-DNA (Figure 5), and the insertion ended with the 3' junction at bp 11,358 within the T-DNA Left Border of the intended insertion (Figure 10A), indicating minor truncations of the T-DNA borders. Right Border and Left Border termini deletions often occur in *Agrobacterium*-mediated transformation (Kim et al., 2007). The number of sequence reads at the 5' and 3' junctions is provided in Table 7. There were no other junctions between the PHP70533 sequences and the maize genome detected in the T1 plant, indicating that there are no additional plasmid-derived insertions present in DP56113 SPTA maintainer. Alignment of the reads to the PHP70533 T-DNA (Figure 10B) and plasmid (Figure 10C) maps shows coverage of the genetic elements found in the intended insertion, along with coverage to the endogenous elements in the T-DNA that were removed by *lox*P recombination (*zm-wus2*, *zm-odp2*, *ubi*ZM1 promoter, 5' UTR, and intron, and *zm-rab17* promoter and 5' UTR). Reads also aligned to the *pin*II terminator elements present on the map in the *zm-wus2*, *zm-odp2*, and *mo-cre* cassettes although these cassettes were removed by recombination. The NGS reads that aligned to these three copies of the *pin*II terminator are from fragments containing the *pin*II terminator in the *DsRed2* cassette of the intended insertion; however, the reads from this single copy align to all copies of the *pin*II terminator in the PHP70533 T-DNA and plasmid maps. There were no unexpected junctions between non-contiguous regions of the intended insertion identified, indicating that there are no rearrangements or additional truncations in the inserted DNA, other than the Right Border and Left Border truncations noted above. Furthermore, there were no junctions between maize genome sequences and the backbone sequence of PHP70533, demonstrating that no plasmid backbone sequences were incorporated into DP56113 SPTA maintainer.

SbS analysis of the T1 generation of DP56113 SPTA maintainer demonstrated that there is a single, intact insertion of the intended insertion derived from the PHP70533 T-DNA in DP56113 SPTA maintainer and that no additional insertions or plasmid backbone sequences are present in its genome.

Table 6. Maize Endogenous Elements in PHP70533 and DP56113 SPTA Maintainer Insertion

Number[1]	Name of Endogenous Element[2]
1	*zm-wus2*
2	*ubi*ZM1 promoter, 5' UTR, and intron
3	*zm-odp2*
4	*zm-rab17* promoter and 5' UTR
5	*In2-1* terminator
6	*zm-aa1* + *zm-bt1* transit peptide
7	*Pg47* promoter
8	*zm-Ms44* promoter
9	*zm-Ms44* artificial microRNA precursor[3]
10	*zm-Ms44* terminator

1. Circled numbers found below linear construct maps in Figures 8-10
2. As shown in the PHP70533 plasmid and T-DNA maps in Figures 3-4 and the intended insertion map in Figure 5.
3. Precursor includes: *zm*-miRNA 5' Precursor 396h, *zm-Ms44* amiRNA Sequence, *zm*-miRNA Precursor 396h, *zm-Ms44* Star Sequence, and *zm*-miRNA 3' Precursor 396h.

Table 7. DP56113 SPTA Maintainer SbS Junction Reads

Description	Supporting Reads at 5' Junction[1]	Unique Reads at 5' Junction[2]	Supporting Reads at 3' Junction[3]	Unique Reads at 3' Junction[4]
DP56113 SPTA Maintainer	87	34	82	32
Control Maize[5]	0	0	0	0
Positive Control[5]	0	0	0	0

1. Total number of sequence reads across the 5' junction of the DP56113 SPTA maintainer insertion.
2. Unique sequence reads establishing the location of the 5' genomic junction of the DP56113 SPTA maintainer insertion at bp 23 of the intended insertion (Figure 5). Multiple identical NGS supporting reads are condensed into each unique read.
3. Total number of sequence reads across the 3' junction of the DP56113 SPTA maintainer insertion.
4. Unique sequence reads establishing the location of the 3' genomic junction of the DP56113 SPTA maintainer insertion at bp 11,358 of the intended insertion (Figure 5). Multiple identical NGS supporting reads are condensed into each unique read.
5. No junctions were detected in either the control maize or positive control samples.

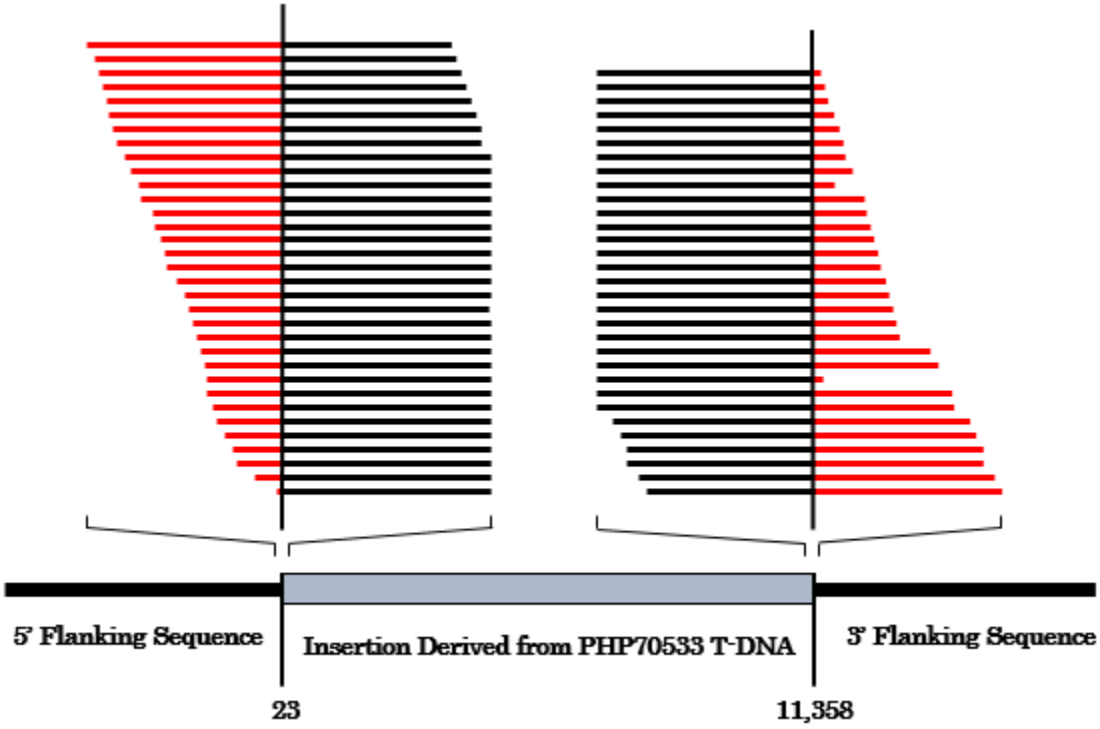


Figure 7. Map of the Insertion in DP56113 SPTA Maintainer

Schematic map of the DNA insertion in DP56113 SPTA maintainer based on the SbS analysis described. The flanking maize genomic regions are indicated in the map. A single copy of the intended insertion derived from the PHP70533 T-DNA, shown by the gray box, is integrated into the maize genome. Vertical lines show the locations of the two-unique genome-plasmid junctions. The numbers below the map indicate the bp location of the junction nucleotide in reference to the sequence of the intended insertion (Figure 5). Representative individual sequencing reads across the junctions are shown as stacked lines above each junction (not to scale); red indicates genomic flanking sequence and black indicates T-DNA sequence within each read.

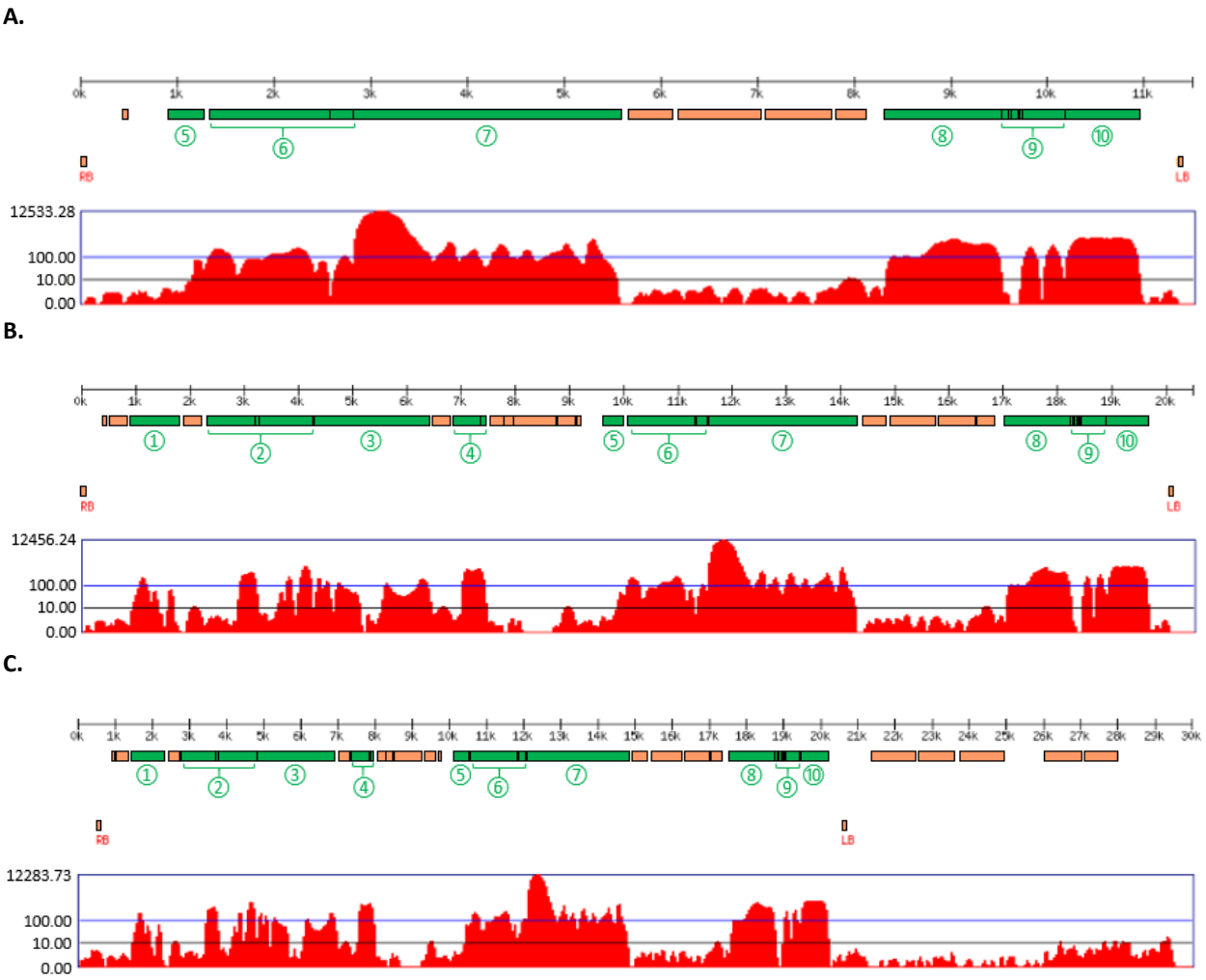


Figure 8. SbS Results for Control Maize

The red coverage graph shows the number of individual NGS reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in plasmid PHP70533 derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. **A)** SbS results for PHH5G control maize aligned against the intended insertion (11,364 bp; Figure 3). Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to some sequence variation between the control maize and the source of the corresponding genetic elements in plasmid PHP70533. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in the PHH5G control maize, and the sequence reads are solely due to the endogenous elements present in the PHH5G genome. **B)** SbS results aligned against the PHP70533 T-DNA originally transformed into maize (20,081 bp). Coverage was

obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid PHP70533 sequence (29,511 bp). Coverage was obtained for the same endogenous elements as in Panel B. The absence of any junctions to the PHP70533 sequence indicates that there are no insertions or backbone sequence present in the PHH5G control maize.

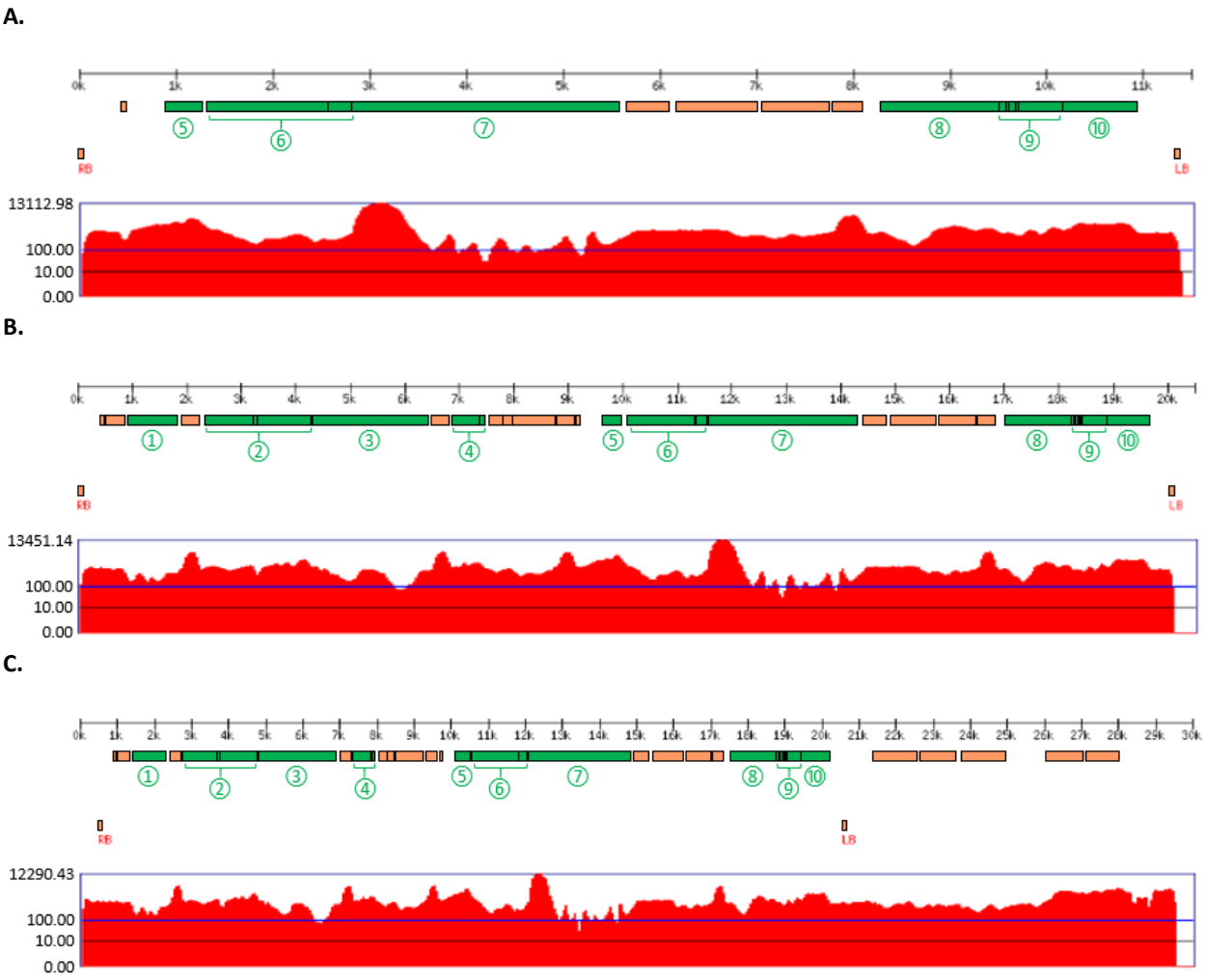


Figure 9. SbS Results for Positive Control Sample

The positive control sample consisted of control maize DNA spiked with PHP70533 plasmid at a level corresponding to one copy of PHP70533 per copy of the maize genome. The red coverage graph shows the number of individual NGS reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in plasmid PHP70533 derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the intended insertion (11,364 bp; Figure 5). Coverage was obtained for the entire intended insertion, indicating efficient capture by the probe library of sequence from the PHP70533 plasmid added to maize genomic DNA. **B)** SbS results aligned against the PHP70533 T-DNA originally transformed into maize (20,081 bp). Coverage was obtained across the full length of the T-DNA. **C)** SbS results aligned against the plasmid PHP70533 sequence (29,511 bp). Coverage was obtained across the full length of the plasmid, again indicating successful capture of PHP70533 sequences by the SbS probe library.

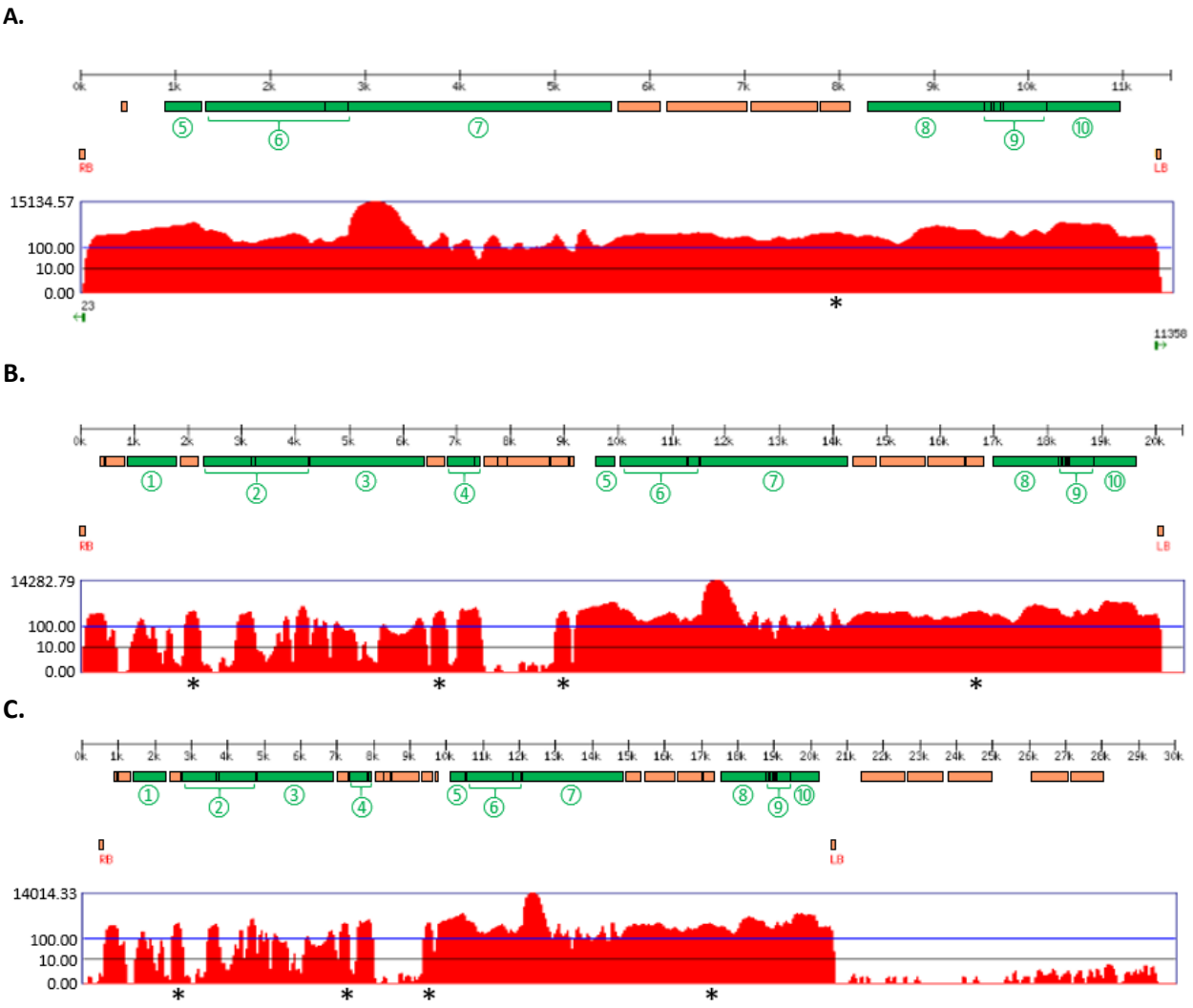


Figure 10. SbS Results for DP56113 SPTA Maintainer

The red coverage graph shows the number of individual NGS reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate endogenous genetic elements in plasmid PHP70533 derived from the maize genome (identified by numbers, Table 6), while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the intended insertion (11,364 bp; Figure 5). Green arrows indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the intended sequence. The insertion comprises bp 23 to 11,358 of the intended insertion shown in Figure 5. The presence of only two junctions demonstrates the presence of a single insertion in the DP56113 SPTA maintainer genome. **B)** SbS results aligned against the PHP70533 T-DNA originally transformed into maize (20,081 bp). Coverage was obtained for the intended insertion from approximately 10k to 21k on the scale bar matching the coverage in Panel A; however, for clarity the junctions identified in Panel A are not

shown in this view. Coverage was also obtained for the endogenous elements in the region from approximately 1k to 10k that was removed by *lox*P recombination, along with all *pin*II terminator elements (*) in the PHP70533 T-DNA due to alignment of reads derived from the *pin*II terminator in the *DsRed2* cassette to the multiple copies of this element in the T-DNA. **C)** SbS results aligned against the entire PHP70533 sequence (29,511 bp). Coverage was obtained for the same elements as described for the PHP70533 T-DNA in Panel B. The absence of any other junctions to the PHP70533 sequence indicates that there are no additional insertions or backbone sequence present in DP56113 SPTA maintainer.

V-C. Southern Blot Analysis of DP56113 SPTA Maintainer

Southern blot analysis was conducted on four generations of DP56113 SPTA maintainer to demonstrate the inserted DNA remained stable across multiple generations. Genomic DNA samples from individual plants of the T1, T2, T3, and T4 generations of DP56113 SPTA maintainer and control maize were analyzed by digestion with restriction enzymes *Bmt* I or *Sca* I. The *Bmt* I-digested genomic DNA samples were hybridized with the *zm-aa1* probe to demonstrate that the 5′ border of the DP56113 SPTA maintainer insertion is intact and remained stable across all four generations of DP56113 SPTA maintainer. Similarly, the *Sca* I-digested genomic DNA samples were hybridized with the *DsRed2* and *Ms44* probes to demonstrate that the 3′ border of the DP56113 SPTA maintainer insertion is intact and remained stable across all four generations of DP56113 SPTA maintainer. The presence of equivalent bands from hybridization with the *zm-aa1*, *DsRed2*, and *Ms44* probes within all four generations analyzed confirmed that the DP56113 SPTA maintainer insertion is stable and equivalent across multiple generations.

Restriction enzymes *Bmt* I and *Sca* I were selected to verify the stability of the DP56113 SPTA maintainer insertion across the T1, T2, T3, and T4 generations of DP56113 SPTA maintainer. Genomic DNA samples from the four generations of DP56113 SPTA maintainer and control maize were digested with either *Bmt* I or *Sca* I and hybridized to probes that detect event-specific fragments from either the 5′ or 3′ genomic border of the insertion. Digestion with *Bmt* I and hybridization with the *zm-aa1* probe would detect the 5′ border, as one *Bmt* I site is in the insertion and the other site is in the flanking genomic sequence (Figure 12). Similarly, digestion with *Sca* I and hybridization with the *DsRed2* or *Ms44* probes would detect the 3′ border of the insertion. Plasmid PHP70533 was added to control maize DNA at a level equivalent to one copy of plasmid per genomic copy and digested with *Bmt* I or *Sca* I, as appropriate, and included on the blot to verify probe hybridization.

The *zm-aa1* and *Ms44* probes are homologous to sequences found in the genome of the control plants and thus additional hybridization bands in all maize samples were expected. These endogenous bands are identified in Table 9 by an asterisk (*) and gray shading.

Hybridization of the *zm-aa1* probe to *Bmt* I-digested genomic DNA resulted in a consistent band of approximately 7,400 bp in all four generations of DP56113 SPTA maintainer (Table 9, Figure 13). In addition to the insertion-derived band, there were endogenous bands of approximately 17,000, 11,000, and 2,600 bp observed in all maize plant samples including the control line (Table 9, Figure 13). These results confirmed that the 5′ border fragment, containing the *zm-aa1* gene in the DP56113 SPTA maintainer insertion, is intact and stable across the four generations of DP56113 SPTA maintainer. The plasmid lanes showed the expected band of 10,901 bp, confirming successful hybridization of the *zm-aa1* probe.

Hybridization of the *DsRed2* probe to *Sca* I-digested genomic DNA resulted in a consistent band of approximately 5,700 bp in all four generations of DP56113 SPTA maintainer (Table 9, Figure 14). These results confirmed that the 3' border fragment, containing the *DsRed2* gene in the DP56113 SPTA maintainer maize insertion, is intact and stable across the four generations of DP56113 SPTA maintainer. The plasmid lanes showed the expected band of 13,511 bp, confirming successful hybridization of the *DsRed2* probe.

Hybridization of the *Ms44* probe to *Sca* I-digested genomic DNA resulted in a consistent band of approximately 5,700 bp in all four generations of DP56113 SPTA maintainer (Table 9, Figure 15), matching the band detected by the *DsRed2* probe. In addition to the insertion-derived band, there was an endogenous band of ~9,400 bp observed in all maize plant samples including the control line (Table 9, Figure 15). These results confirmed that the 3' border fragment, containing the *Ms44* cassette in the DP56113 SPTA maintainer insertion, is intact and stable across the four generations of DP56113 SPTA maintainer. The plasmid lanes showed the expected band of 13,511 bp, confirming successful hybridization of the *Ms44* probe.

Southern blot analysis with digests specific for the 5' and 3' borders, and hybridization with the *zm-aa1*, *DsRed2*, or *Ms44* probes, showed that the 5' and 3' genomic borders of the DP56113 SPTA maintainer insertion are intact and stable across four generations of DP56113 SPTA maintainer during the breeding process.

Table 8. Description of DNA Probes Used for Southern Hybridization of DP56113 SPTA Maintainer

Probe Name	Probe Lot#	Position on Plasmid (bp to bp)[a]	Position on DP56113 SPTA Maintainer Intended Insertion (bp to bp)[b]	Length (bp)
zm-aa1	19-DP-08	10,040 to 11,302	1,323 to 2,585	1263
DsRed2	19-DP-06	15,790 to 16,467	7,073 to 7,750	678
Ms44	19-DP-07	18,230 to 18,863	9,513 to 10,146	634

[a] Positions are based on plasmid map of PHP70533 (Figure 11).
[b] Positions are based on map of the intended insertion in DP56113 SPTA maintainer (Figure 12).

Table 9. Predicted and Observed Hybridizing Bands on Southern Blots of DP56113 SPTA Maintainer

Enzyme	Probe	Predicted Band Size from Plasmid[a] (bp)	Predicted Band Size from Intended Insertion[b] (bp)	Observed Fragment Size[c] (bp)	Figure
Bmt I	*zm-aa1*	10,901	>6,800	~7,400 ~17,000* ~11,000* ~2,600*	13
Sca I	*DsRed2*	13,511	>5,100	~5,700	14
	Ms44	13,511	>5,100	~5,700 ~9,400*	15

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences. These bands were identified in the maize control line that was analyzed.
[a] Predicted size for hybridizing fragment based on *Bmt* I or *Sca* I digestion and the plasmid map of PHP70533 (Figure 11).
[b] Predicted size for hybridization in genomic DNA samples is based on the DP56113 SPTA maintainer intended insertion map (Figure 12). The sizes are rounded to the nearest 100 bp for fragments where one of the restriction enzyme sites is expected in the maize genome.
[c] Observed size for hybridizing bands are approximated from the DIG-labeled DNA Molecular Weight Markers II and VII on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.

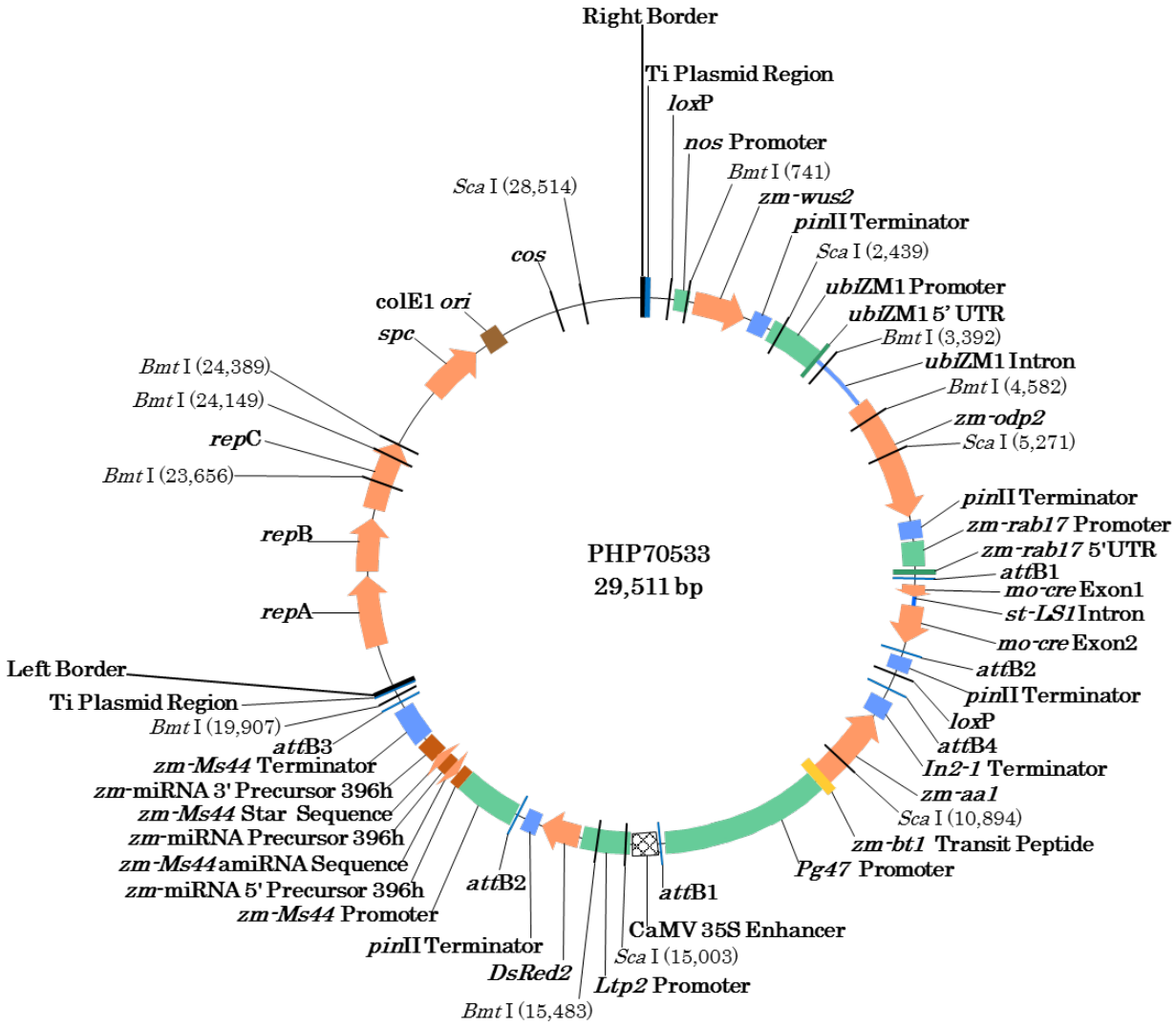


Figure 11. Schematic Map of Plasmid PHP70533 with Restriction Sites for Southern Blot Analysis

Schematic map of the plasmid PHP70533 containing the *zm-wus2, zm-odp2, mo-cre, zm-aa1,* and *DsRed2* gene cassettes and the *zm-Ms44* amiRNA cassette. The plasmid size is 29,511 bp. Right and Left Borders flank the T-DNA (Figure 12) that was inserted into the plant genome during transformation. The *Bmt* I and *Sca* I restriction enzyme sites used for Southern blot analysis are indicated.

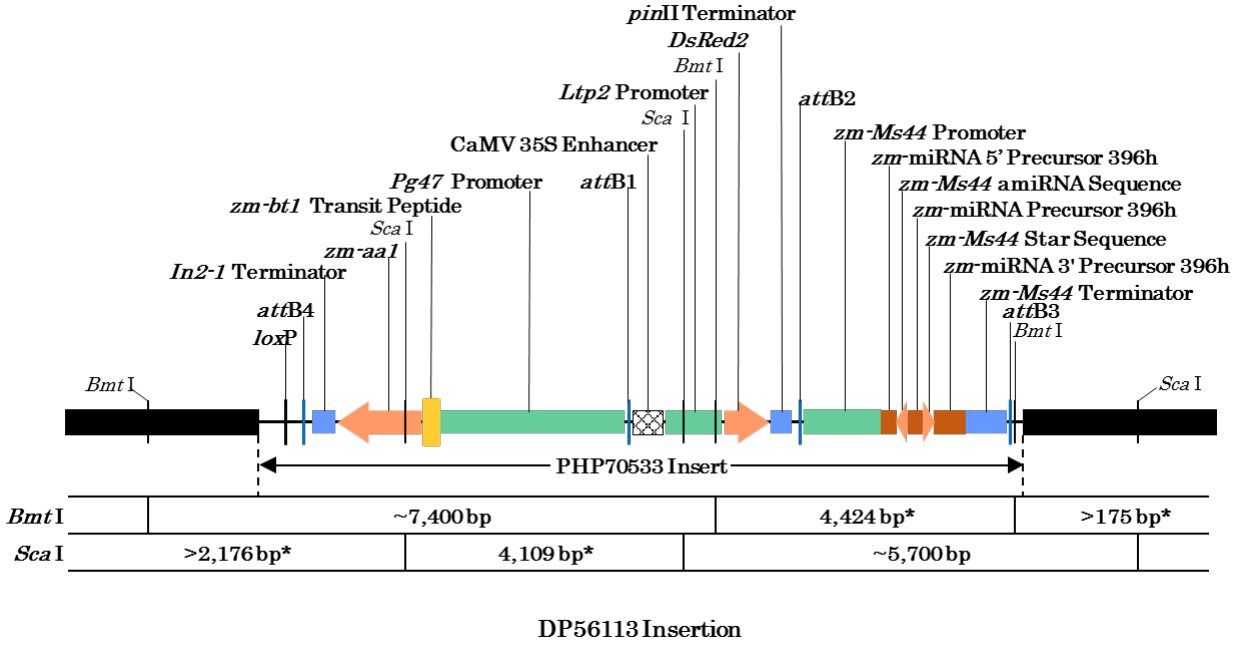


Figure 12. Map of DP56113 SPTA Maintainer Insertion with Southern Restriction Enzyme Fragments

Schematic map of the DP56113 SPTA maintainer insertion indicating the *Bmt* I and *Sca* I restriction enzyme sites. The PHP70533 insert in DP56113 SPTA maintainer is marked and the vertical dash lines indicate the genomic border/insert junctions. The flanking maize genomic regions are represented by horizontal black bars. Restriction sites used in Southern analysis are indicated with the size of the observed fragment shown below the map in base pairs (bp), approximated to the nearest 100 bp. Fragments marked with an asterisk (*) are predicted from the intended insertion map (Figure 5) but were not detected with the restriction enzyme and probe combinations used in the study.

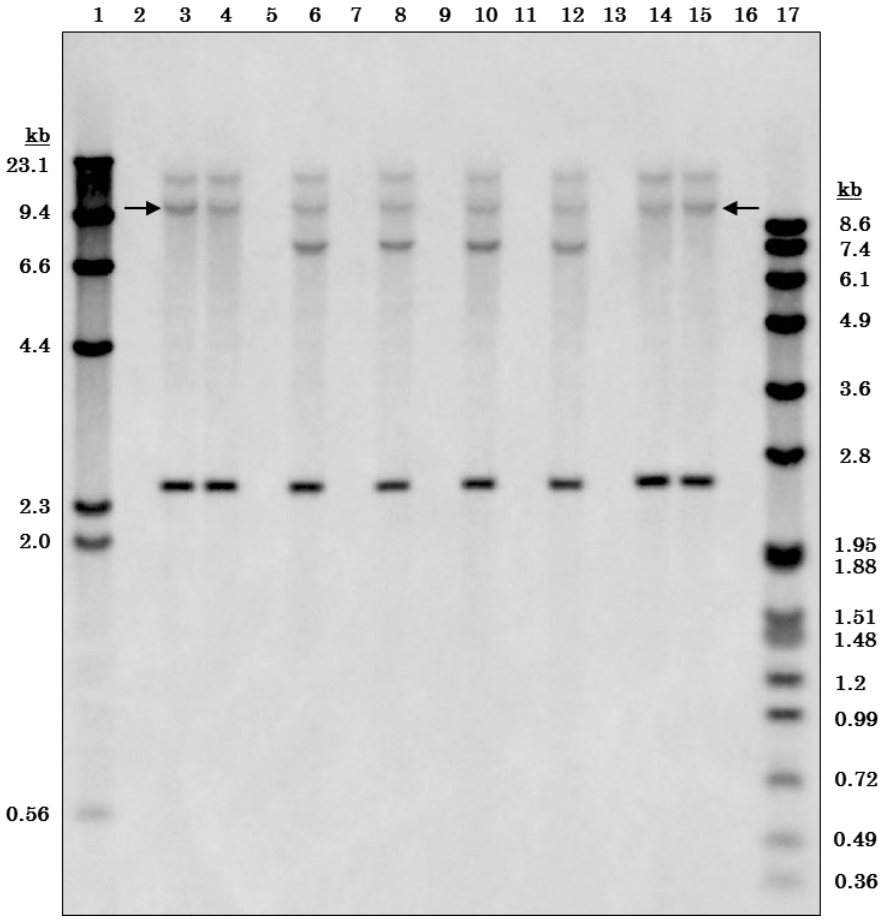


Figure 13. Southern Blot Analysis of DP56113 SPTA Maintainer: *Bmt* I Digest with *zm-aa1* Probe

Lane	Sample	Lane	Sample
1	DIG II marker	10	DP56113 SPTA maintainer T1 generation
2	Blank	11	Blank
3	1 copy PHP70533 + PHH5G control	12	DP56113 SPTA maintainer T1 generation
4	PHH5G control	13	Blank
5	Blank	14	PHH5G control
6	DP56113 SPTA maintainer T1 generation	15	Blank
7	Blank	16	1 copy PHP70533 + PHH5G control
8	DP56113 SPTA maintainer T2 generation	17	DIG VII marker
9	Blank		

Genomic DNA isolated from leaf tissue of the T1, T2, T3, and T4 generations of DP56113 SPTA maintainer plants and control maize plants was digested with *Bmt* I and hybridized with the *zm-aa1* probe. Approximately 3 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP70533 at approximately one gene copy number and 3 µg of control maize DNA. Sizes of the DIG II and DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). **Note:** The plasmid band at 10,901 bp migrates

equivalently with the ~11,000 bp endogenous band but can be detected by increased intensity in lanes 3 and 15 (arrows).

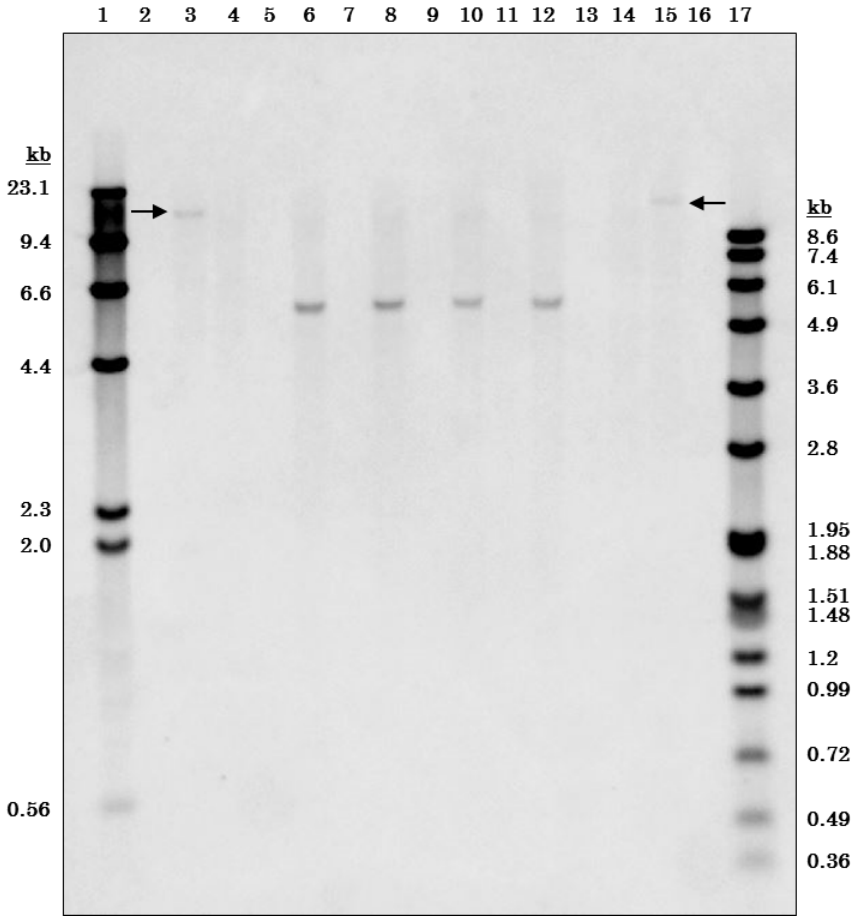


Figure 14. Southern Blot Analysis of DP56113 SPTA Maintainer: *Sca* I Digest with *DsRed2* Probe

Lane	Sample	Lane	Sample
1	DIG II marker	10	DP56113 SPTA maintainer T1 generation
2	Blank	11	Blank
3	1 copy PHP70533 + PHH5G control	12	DP56113 SPTA maintainer T1 generation
4	PHH5G control	13	Blank
5	Blank	14	PHH5G control
6	DP56113 SPTA maintainer T1 generation	15	Blank
7	Blank	16	1 copy PHP70533 + PHH5G control
8	DP56113 SPTA maintainer T2 generation	17	DIG VII marker
9	Blank		

Genomic DNA isolated from leaf tissue of the T1, T2, T3, and T4 generations of DP56113 SPTA maintainer plants and control maize plants was digested with *Sca* I and hybridized with the *DsRed2* probe. Approximately 3 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP70533 at approximately one gene copy number and 3 µg of control maize DNA. Sizes of the DIG II and DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). **Note:** The plasmid band at 13,511 bp is indicated by the arrows in lanes 3 and 15.

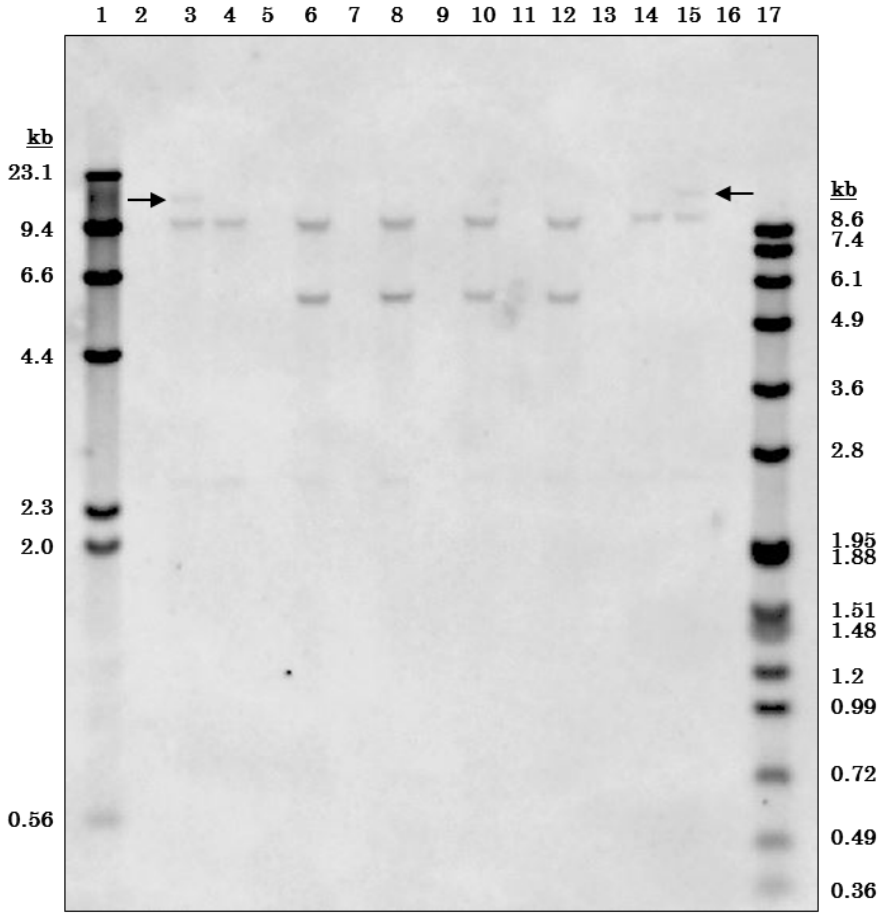


Figure 15. Southern Blot Analysis of DP56113 SPTA Maintainer: *Sca* I Digest with *Ms44* Probe

Lane	Sample	Lane	Sample
1	DIG II marker	10	DP56113 SPTA maintainer T1 generation
2	Blank	11	Blank
3	1 copy PHP70533 + PHH5G control	12	DP56113 SPTA maintainer T1 generation
4	PHH5G control	13	Blank
5	Blank	14	PHH5G control
6	DP56113 SPTA maintainer T1 generation	15	Blank
7	Blank	16	1 copy PHP70533 + PHH5G control
8	DP56113 SPTA maintainer T2 generation	17	DIG VII marker
9	Blank		

Genomic DNA isolated from leaf tissue of the T1, T2, T3, and T4 generations of DP56113 SPTA maintainer plants and control maize plants was digested with *Sca* I and hybridized with the *Ms44* probe. Approximately 3 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid PHP70533 at approximately one gene copy number and 3 µg of control maize DNA. Sizes of the DIG II and DIG VII molecular weight markers are indicated adjacent to the blot image in kilobases (kb). **Note:** The plasmid band at 13,511 bp is indicated by the arrows in lanes 3 and 15.

V-D. Open Reading Frame Analysis of DP56113 SPTA Maintainer

The potential allergenic cross-reactivity of the translated ORFs at the DP56113 SPTA maintainer insertion site was assessed by comparison of their sequences to the sequences in the Comprehensive Protein Allergen Resource (COMPARE) database. The COMPARE database (January 2019), compiled through a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins, and Bioinformatics (PATB) Committee, is peer-reviewed and contains 2,081 sequences. A separate search was performed to find any contiguous 8-residue exact matches between the translated ORF sequences and the sequences in the COMPARE database.

The potential toxicity of the translated ORFs at the DP56113 SPTA maintainer insertion site was assessed by comparison of their sequences to the sequences in an internally-developed protein toxin database. The internally-developed toxin database is updated annually and was last updated in January 2019.

One of the translated ORFs at the DP56113 SPTA maintainer insertion site corresponding to the intended ZM-AA1 (alpha-amylase) protein, returned an above-threshold alignment (87.6%) from the search against the COMPARE database. This alignment does not include the ZM-AA1 protein transit peptide sequence and is to an unnamed protein product with alpha-amylase activity (GenBank accession BAG93480.1) from rice (*Oryza sativa*).

The up-regulated alpha-amylase in DP56113 SPTA maintainer is not new to maize (Hirano *et al.*, 2016; Lombard *et al.*, 2014). The ZM-AA1 protein is native to maize and has cross-reactivity with other allergens already in pollen (maize and Poaceae in general) (Besler *et al.*, 2001; Lombard *et al.*, 2014), and for those that have allergens to maize pollen, the precautions against sensitivity and allergenic reactions would be unchanged. Maize is not considered allergenic, and the expression of the ZM-AA1 protein in DP56113 SPTA maintainer maize is not likely to change its allergenic potential.

None of the translated ORFs at the DP56113 SPTA maintainer insertion site returned alignments from the search against the internally-developed toxin database, indicating that DP56113 SPTA maintainer is unlikely to be a human or animal toxin.

V-E. Inheritance and Genetic Stability of the Introduced Traits in DP56113 SPTA Maintainer

The stability of the inserted DNA during the breeding process is evaluated by examining the inheritance and segregation of the genes and/or traits in multiple generations. The segregation

of these genes and/or traits as a single unit and as a single genetic locus confirms that the inserted DNA is predictably and stably inherited through the conventional breeding process.

The inheritance pattern of the T-DNA insert within DP56113 SPTA maintainer was investigated by determining segregation of the *zm-Ms44* amiRNA, and *zm-aa1* and *DsRed2* genes within five generations (T2, T3, T4, T5, and F1; Figure 2) representing a range of different crossing, backcrossing, and selfing points in a typical maize breeding program. Leaf punches from individual plants of each generation were analyzed for the presence of the PHP70533 T-DNA insert by event-specific and for the presence of each of the introduced genes by gene-specific PCR qualitative polymerase chain reaction (qPCR). Statistical analysis (chi-square test at 0.05 significance level) was conducted to compare the observed segregation ratio to the expected segregation ratio of 1:1 for each generation.

Results from the segregation analysis are provided in Table 10. In every case, a positive plant tested positive for the presence of the DP56113 SPTA maintainer insertion; and the *zm-Ms44* amiRNA, and *zm-aa1* and *DsRed2* genes indicating that the inserted T-DNA and its genetic elements within DP56113 SPTA maintainer segregated together. A chi-square (χ^2) analysis was performed on the data, and no statistically significant differences were found between the observed and expected segregation ratios for each of the T2, T3, T4, T5, and F1 generations of DP56113 SPTA maintainer (Table 10). Results indicated that within these five generations, each of the introduced genes segregated according to Mendelian rules of inheritance for a single genetic locus. These results were consistent with SbS and Southern analysis data indicating the stable integration of the insert at a single site in the genome and stable genetic inheritance of the DNA insertion of DP56113 SPTA maintainer across breeding generations. Materials and methods for the multi-generation segregation analysis are described in Appendix 4.

Table 10. Summary of Genotypic and Phenotypic Results for Segregating Generations of DP56113 SPTA Maintainer

Generation	Expected Segregation Ratio	Observed Segregation			Statistical Analysis	
	(Positive:Negative)	Positive	Negative	Total	Chi-Square[a]	P-Value
T2	1:1	50	50	100	0.00	1.0000
T3	1:1	48	52	100	0.16	0.6892
T4	1:1	48	52	100	0.16	0.6892
T5	1:1	46	54	100	0.64	0.4237
F1	1:1	49	51	100	0.04	0.8415

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

V-F. Conclusions on Molecular Characterization and Genetic Stability of DP56113 SPTA Maintainer

SbS and Southern blot analyses were conducted to characterize the DNA insertion in DP56113 SPTA maintainer. SbS analysis confirmed that a single, intact PHP70533 T-DNA was inserted into the maize genome and the integrity of the inserted DNA was maintained. Southern blot analysis on four generations of DP56113 SPTA maintainer confirmed the stability of inheritance of the DNA insertion during traditional breeding procedures. SbS analysis results also showed no plasmid backbone sequences were incorporated into DP56113 SPTA maintainer

Bioinformatics assessment of translated ORFs at the DP56113 SPTA maintainer insertion site found no similarity to known toxins.

The allergenicity assessment of translated ORFs identified an unidentified rice (*Oryza sativa*) amino acid sequence with an 88% identity homology over the α-amylase portion (excluding the transit peptide) of the ZM-AA1 protein in DP56113 SPTA maintainer. The up-regulated alpha amylase is not new (Hirano *et al.*, 2016; Lombard *et al.*, 2014), it has cross-reactivity with other allergens already in pollen (maize and Poaceae in general) (Besler *et al.*, 2001; Lombard *et al.*, 2014), and for those that have allergens to maize pollen, the precautions against sensitivity and allergenic reactions would be unchanged. The ZM-AA1 protein is native to maize, maize is not a common allergenic food and the modification of DP56113 SPTA maintainer is not expected to alter the allergenic potential of maize as a food source.

The inheritance and genetic stability of the inserted DNA was confirmed in five generations of DP56113 SPTA maintainer. The results of this analysis were consistent with the finding of a single locus of the DP56113 SPTA maintainer insertion that segregated according to Mendelian rules of inheritance. The stability of the insertion and of the herbicide resistance phenotype was demonstrated in these populations.

Together, these analyses demonstrated the presence of a single, intact, stable T-DNA insertion, with no plasmid backbone sequences, and that the DP56113 SPTA maintainer is unlikely to alter the potential for human or animal allergenicity or toxicity.

VI. Characterization of the Proteins Introduced into DP56113 SPTA Maintainer

VI-A. Identity and Function of the ZM-AA1 and DsRed2 Proteins Present in DP56113 SPTA Maintainer

VI-A.1. ZM-AA1 Protein

The *zm-aa1* gene cassette in DP56113 SPTA maintainer contains a truncated version of the maize α-amylase (*zm-aa1*) gene (Schnable et al., 2009) encoding the ZM-AA1 protein. The *zm-aa1* coding region is preceded by the sequence encoding the transit peptide from the maize amyloplast membrane protein *Brittle-1* (*zm-bt1*) gene (Sullivan et al., 1991) that targets the ZM-AA1 protein to the amyloplast. The ZM-AA1 protein contributes to altered starch content ((Chao and Scandalios, 1971) and prevents accumulation of starch in the nascent pollen grain, thus preventing the pollen from developing and germinating normally and rendering the pollen infertile. The complete translation product, including transit peptide, is 495 amino acids in length and has a molecular weight of approximately 54 kDa (Figure 16). The processed ZM-AA1 protein, with the transit peptide removed, is 420 amino acids in length and has a molecular weight of approximately 46 kDa (Figure 16). The processed ZM-AA1 protein differs from the native protein in that it lacks the 21 N-terminal amino acid residues found in the native protein, including the initial methionine residue. The ZM-AA1 protein in DP56113 SPTA maintainer is identical to the protein in the antecedent organism, 32138 SPT maintainer.

```
  1   MAATMAVTTM VTRSKESWSS LQVPAVAFPW KPRGGKTGGL EFPRRAMFAS
 51   VGLNVCPGVP AGRDPREPDP KVVRAACGLV QAQVLFQGFN WESCKQQGGW
101   YNRLKAQVDD IAKAGVTHVW LPPPSHSVSP QGYMPGRLYD LDASKYGTAA
151   ELKSLIAAFH GRGVQCVADI VINHRCAEKK DARGVYCIFE GGTPDDRLDW
201   GPGMICSDDT QYSDGTGHRD TGEGFAAAPD IDHLNPRVQR ELSAWLNWLR
251   SDAVGFDGWR LDFAKGYSPA VARMYVESTG PPSFVVAEIW NSLSYSGDGK
301   PAPNQDQCRQ ELLDWTRAVG GPAMAFDFPT KGLLQAGVQG ELWRLRDSSG
351   NAAGLIGWAP EKAVTFVDNH DTGSTQKLWP FPSDKVMQGY AYILTHPGVP
401   CIFYDHMFDW NLKQEISTLS AIRARNGIRA GSKLRILVAD ADAYVAVVDE
451   KVMVKIGTRY GVSSVVPSDF HPAAHGKDYC VWEKASLRVP AGRHL*
```

Figure 16. Deduced Amino Acid Sequence of the ZM-AA1 Protein

The deduced amino acid sequence from translation of the *zm-bt1* transit peptide and the *zm-aa1* coding regions from plasmid PHP70533. The asterisk (*) indicates the translational stop codon. The complete translation product, including the transit peptide (underlined region), is 495 amino acids in length and has an approximate weight of 54 kDa. The processed ZM-AA1 protein, with the transit peptide removed, is 420 amino acids in length and has an approximate weight of 46 kDa.

VI-A.1A. ZM-AA1 Protein Function and Activity

Starch molecules are glucose polymers linked together by α -1,4 and α -1,6 glucosidic bonds (Figure 17). α -amylases belong to a family of glycosyl-hydrolases (1,4-α-D-Glucan glucanohydrolase) catalyzing hydrolysis of (1-4)-α-D-glucosidic linkages in polysaccharide molecules, such as starch. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose, maltose and/or, glucose. Expression of ZM-AA1 in the developing pollen of DP56113 SPTA maintainer results in the depletion of starch and deprivation of the energy reserves required for pollen germination and fertilization, thereby resulting in pollen infertility.

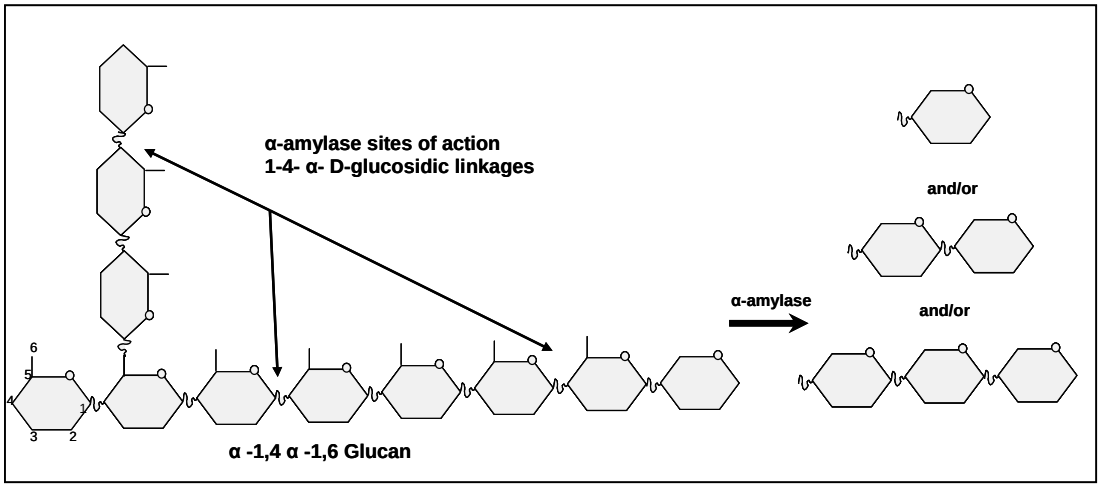
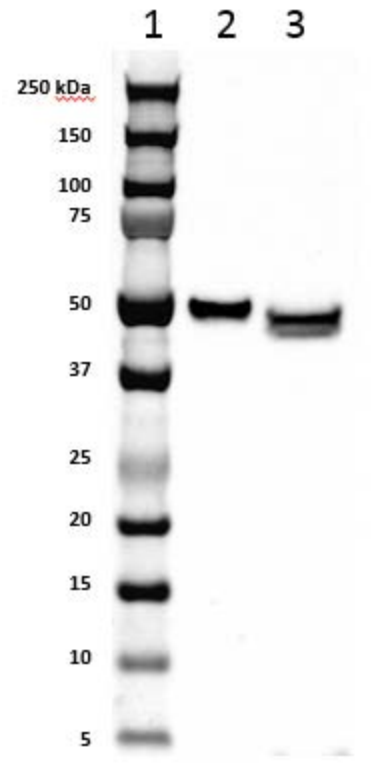


Figure 17. α-Amylase Mode of Action Diagram

VI-A.1B. Characterization of the Introduced ZM-AA1 Protein in DP56113 SPTA Maintainer and Microbially-Produced Reference Standard ZM-AA1 Protein

Western blot analysis demonstrated that the ZM-AA1 protein was immunoreactive to a ZM-AA1 monoclonal antibody and visible as a band consistent with the expected molecular weight of approximately 50 kDa (Figure 18).



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	ZM-AA1 Analytical Standard (100 ng)
3	DP56113 SPTA Maintainer -Derived ZM-AA1 Protein

Note: kilodalton (kDa), nanogram (ng).

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

Figure 18. Western Blot Results for ZM-AA1 Protein Derived from DP56113 SPTA Maintainer

VI-A.1C. Conclusion of Analysis of Amino Acid Sequence Alignment and Western Blot Analysis of the ZM-AA1 Protein

The ZM-AA1 protein derived from DP56113 SPTA maintainer had the expected molecular weight and immunoreactivity.

VI-A.1D. Safety of the ZM-AA1 Protein in DP56113 SPTA Maintainer

The α-amylase expressed in DP56113 SPTA maintainer is derived from two endogenous maize gene sequences that encode the ZM-AA1 protein, and the Brittle-1 transit peptide required for directing the ZM-AA1 protein into the amyloplasts. Endogenous *zm-aa1* gene is predominantly expressed in the scutellum tissues of the germinating seed and minimally expressed (<0.25% of

the levels in the scutellum) in endosperm, stalk, leaf and root. Expression of the endogenous *zm-aa1* in the scutellum tissues corresponds well with the literature evidence that α-amylases are required during cereal seed germination are required for the hydrolysis of endosperm starch into metabolizable sugars, which provide the energy for the growth of roots and shoots (Akazawa and Hara-Nishimura, 1985; Beck and Ziegler, 1989).

Germinating maize seeds, known as sprouts, are extensively used in Latin America in the production of alcoholic beverages, for example, Chicha (maize beer), Sora, Napú, Fubá, Champuz, Acupe, and Tesgüino (Lorence-Quiñones et al., 1999). Chicha is the most important traditional fermented beverage in Latin America and is made by germinating maize seed to produce amylase required for starch conversion. Chicha has been consumed by the Andean Indians for centuries. Sprouted maize seed has been increasingly used in bread and tortilla products (see, for example, www.foodforlife.com) and has been explored as a feed supplement (Naga, 1986). In addition, livestock feeding of maize grain that has sprouted from moisture exposure is an accepted practice (Heiniger, 1999). Therefore, there is a previous history of exposure and safe use of ZM-AA1 α-amylase endogenous to the germinating maize seed.

The α-amylases are ubiquitous enzymes present in many organisms including plants and insects (Campos et al., 1989; Cristofoletti et al., 2001; Raimbaud et al., 1989; Silva et al., 1999). The α-amylases from multiple sources including plants, fungi and bacteria have a long history of safe consumption in foods. Purified microbial α-amylases are commonly used in the modern food processing industry (Pariza and Johnson, 2001) and these enzymes have a long history of safe use. Additionally, their safety has been verified by an array of laboratory studies (see, for example, Landry et al., 2003).

In plants, α-amylases aid in the hydrolysis of transient starch in the leaves, which occurs during the dark cycle of photosynthesis, and are also required for the hydrolysis of storage starch during seed germination or tuber sprouting. Multiple forms of α-amylases exist in maize, and are expressed in the endosperm and other tissues (Chao and Scandalios, 1971; James et al., 2007). Amylases are reported to occur endogenously in the pollen of numerous plants (Bhadula and Sawhney, 1989; Castro and Clément, 2007), including maize, where starch hydrolysis is associated with pollen germination (Agarwala et al., 1981; Brewbaker, 1971; Wakhle et al., 1983). Castro and Clément (2007) demonstrated that the amylase activity in *Lilium* pollen is attributed, in part, to α-amylase protein.

Maize has a long history of safe food and feed use for humans and animals. Maize is not considered a common allergenic food (Hefle et al., 1996; Moneret-Vautrin et al., 1998), although in a few case studies, allergenic reactions were reported and maize allergens identified (Pasini et al., 2002; Pastorello et al., 2000; Pastorello et al., 2003; Weichel et al., 2006). .

As stated in section VI-D, a bioinformatics assessment of translated ORFs at the DP56113 SPTA maintainer insertion site found no similarity to known toxins.

The allergenicity assessment of translated ORFs identified an unidentified rice (*Oryza sativa*) amino acid sequence with an 88% identity homology over the α-amylase portion (excluding the transit peptide) of the ZM-AA1 protein in DP56113 SPTA maintainer. The up-regulated alpha amylase is not new (Lombard *et al.* 2014; Hirano *et al*., 2016);, it has cross-reactivity with other allergens already in pollen (maize and Poaceae in general) (Besler *et al.*, 2001; Lombard *et al.*, 2014), and for those that have allergens to maize pollen, the precautions against sensitivity and allergenic reactions would be unchanged. The ZM-AA1 protein is native to maize, maize is not a common allergenic food and the modification of DP56113 SPTA maintainer is not expected to alter the allergenic potential of maize as a food source.

Based on this information, Pioneer determines that allergen and toxin risks from the ZM-AA1 protein are negligible in DP56113 SPTA maintainer.

VI-A.2. DsRed2 Protein

The *DsRed2* gene cassette in DP56113 SPTA maintainer contains a modified version of the *Discosoma sp.* (coral anemone) red fluorescent protein (*DsRed2*) gene (Clontech, 2001), in which an internal *Bst*E II restriction site was removed from the original *DsRed2* gene without altering the amino acid sequence of the expressed protein. The tissue-specific expression of the DsRed2 protein in the aleurone layer of the maize seed produces a red coloration in seeds that contain the DNA insertion, allowing for differentiation during seed sorting. The DsRed2 protein is 225 amino acids in length and has a molecular weight of approximately 26 kDa (Figure 19). The DsRed2 protein in DP56113 SPTA maintainer is identical to the DsRed2 protein in the antecedent organism 32138 SPT maintainer.

```
  1   MASSENVITE FMRFKVRMEG TVNGHEFEIE GEGEGRPYEG HNTVKLKVTK
 51   GGPLPFAWDI LSPQFQYGSK VYVKHPADIP DYKKLSFPEG FKWERVMNFE
101   DGGVATVTQD SSLQDGCFIY KVKFIGVNFP SDGPVMQKKT MGWEASTERL
151   YPRDGVLKGE THKALKLKDG GHYLVEFKSI YMAKKPVQLP GYYYVDAKLD
201   ITSHNEDYTI VEQYERTEGR HHLFL*
```

Figure 19. Deduced Amino Acid Sequence of the DS-RED2 Protein

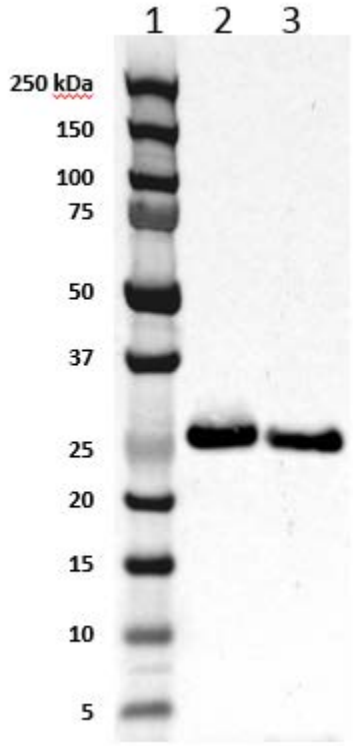
The deduced amino acid sequence from translation of the *DsRed2* gene from plasmid PHP70533. The asterisk (*) indicates the translational stop codon. The full-length protein is 225 amino acids in length and has an approximate weight of 26 kDa.

VI-A.2A. DsRed2 Protein Function and Activity

The DsRed2 protein belongs to a family of red fluorescent proteins which are members of a larger group of proteins in several *Anthozoa* species (this class includes corals, anemones and sea pens). The unique feature of fluorescent proteins is their ability to enter an excited state and emit light of a certain wavelength (*i.e.* fluoresce) upon absorption of ultraviolet or visible light. Application of the DsRed2 protein as a visual selection marker for high-throughput seed sorting has been previously demonstrated in *Arabidopsis* (Stuitje et al., 2003). The DsRed2 protein provides a very effective means of tracking GE maize seed expressing this protein and is used as a quality assurance color marker that allows monitoring of seeds to ensure seed purity.

VI-A.2B. Characterization and Equivalence of the DS-Red2 Protein in DP56113 SPTA Maintainer to a Microbially-Produced Reference Standard DsRed2 Protein

Western blot analysis demonstrated that the DsRed2 protein was immunoreactive to a DsRed2 polyclonal antibody and visible as a band consistent with the expected molecular weight of approximately 26 kDa (Figure 20).



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	DsRed2 Analytical Standard (100 ng)
3	DP56113 SPTA Maintainer -Derived DsRed2 Protein

Note: kilodalton (kDa), nanogram (ng).

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

Figure 20. Western Blot Results for DsRed2 Protein Derived from DP56113 SPTA Maintainer

VI-A.2C. Conclusion of Analysis of Amino Acid Sequence Alignment and Western Blot Analysis of the DsRed2 Protein

The DsRed2 protein derived from DP56113 SPTA maintainer had the expected molecular weight and immunoreactivity.

VI-A.2D. Safety of the DsRed2 Protein in DP56113 SPTA Maintainer

A food and feed safety assessment was conducted to assess the allergenicity and toxicity potential of the DsRed2 protein. The data and assessment form the basis of the conclusion that the DsRed2 protein is unlikely to be toxic or allergenic to humans or animals, and therefore is safe for human and animal consumption. The conclusions of the safety assessment are summarized below.

Toxicological Safety Assessment

The DsRed2 protein belongs to a family of red fluorescent proteins (RFP) which are members of a group of fluorescent proteins identified in several *Anthozoa* species. The DsRed2 protein is a modified variant of the original RFP isolated from a coral-like anemone *Discosoma* sp. Although no evidence could be found regarding the specific use of the DsRed2 protein in the food industry, a DsRed2 variant has been successfully used as a vital marker in mice. There were no observed detrimental effects (Long et al., 2005), or signs of toxicity (Figueiredo et al., 2008), when DsRed2 variants were over expressed in mice.

Plants stably expressing DsRed2 variants did not show any abnormalities, indicating that the protein did not interfere with plant growth, development, fertility, germination or morphogenesis (Dietrich and Maiss, 2002; Jach et al., 2001; Mirabella et al., 2004; Stuitje et al., 2003; Wenck et al., 2003).

The potential toxicity of the DsRed2 protein was assessed by comparison of its sequence to the sequences in an internal toxin database that is updated annually. The internal toxin database is a subset of sequences found in UniProtKB/Swiss-Prot (http://www.uniprot.org/), where the proteins in UniProtKB/Swiss-Prot are filtered for molecular function by keywords that could imply toxicity or adverse health effects (*e.g.*, toxin, hemagglutinin, vasoactive, etc.). The search was conducted with BLASTP using default parameters, except that the *E*-value was set to 0.0001 and all of the alignments at or below the *E*-value threshold were returned.

There was no evidence of acute toxicity in mice at a target dose of 2000 mg per kg of body weight (equivalent to 1860 mg of DsRed2 protein). To translate this type of mouse acute toxicity dose to human exposure, based on expression levels of the DsRed2 protein in DP56113 SPTA maintainer seed, a child weighing 10 kg would have to consume approximately 39 kg of DP56113 SPTA maintainer seed and an adult weighing approximately 60 kg would have to consume about 232 kg of DP56113 SPTA maintainer seed. Based on these simplistic calculations, there is a clear margin of safety for the DsRed2 protein, especially considering other factors such as the low likelihood of exposure to this protein due to its use as part of an internal seed production process.

No alignments were returned between the DsRed2 protein and any protein sequence in the internal toxin database. Therefore, no toxicity concerns arose from the bioinformatics

assessment of the DsRed2 protein. Additionally, no evidence of toxicity has been observed in studies that have been conducted using RFPs or related green fluorescent proteins in GE plants and animals (Richards et al., 2003). These data support the conclusion that the DsRed2 protein is unlikely to be a toxin.

Allergenicity Assessment

The DsRed2 protein was evaluated for its allergenicity potential using FDA's published guidance for the early food safety evaluation of new proteins in new plant varieties (US-FDA, 2006). The allergenic potential of the DsRed2 protein was assessed by: 1) bioinformatic comparison of the amino acid sequence of the DsRed2 protein with known or putative protein allergen sequences; 2) evaluation of the stability of the DsRed2 protein using an *in vitro* gastric digestion model; and 3) assessment of the DsRed2 gene source and history of use or exposure.

Two separate bioinformatics searches for the Ds-Red2 protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2019 database (January 2019) available at http://comparedatabase.org. This peer-reviewed database is a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins, and Bioinformatics (PATB) Committee and is comprised of 2,081 sequences. The first search used the Ds-Red2 protein sequence as the query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the allergen sequences. The search was conducted using default parameters, except the E-score threshold was set to 10^{-4}. An E-score threshold of 10^{-4} has been shown to be an appropriate value for allergenicity searches (Mirsky et al., 2013). The generated alignments were examined to identify any that are a length of 80 or greater and possess a sequence identity of ≥ 35%. The second search used an in-house Perl script to identify any contiguous 8-residue identical matches between the Ds-Red2 protein sequence and the allergen sequences. Results of the search of the Ds-Red2 protein sequence against the COMPARE database of known and putative allergen sequences found no alignments that were a length of 80 or greater with a sequence identity of ≥ 35%. No contiguous 8 residue matches between the Ds-Red2 protein sequence and the allergen sequences were identified in the second search. Taken together, the comparisons of the Ds-Red2 protein sequence to the allergen sequences showed that there is no apparent allergenicity concern regarding the Ds-Red2 protein.

The DsRed2 protein was rapidly hydrolyzed (less than 30 seconds) in simulated gastric fluid containing pepsin at pH 1.2 as demonstrated by SDS-PAGE analysis. Finally, the DsRed2 protein is not glycosylated.

The DsRed2 protein is used as a marker that enables efficient detection and removal of maize seed that express the DsRed2 protein. Results of the safety assessment indicate that the DsRed2 protein is safe for animal and human consumption. Based on the data and information provided

in this submission, we have determined that the DsRed2 protein is unlikely to be toxic or allergenic to humans or animals.

VI-B. Concentration of ZM-AA1 and DsRed2 Proteins in DP56113 SPTA Maintainer

The values of ZM-AA1 and DsRed2 protein mean concentrations in leaf, root, and whole-plant (including forage) tissues over the course of the growing season, as well as the mean concentrations in pollen and seed, are summarized in Tables 11 and 12, respectively, for DP56113 SPTA maintainer.

The ZM-AA1 and DsRed2 proteins in DP56113 SPTA maintainer are identical, expressed as expected, and function similarly to those in the 32138 SPT maintainer antecedent organism.

Table 11. Across-Site Summary of Expressed Trait ZM-AA1 Protein Concentrations of DP56113 SPTA Maintainer

Tissue (Growth Stage)	ng ZM-AA1/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	Range	Standard Deviation	Sample LLOQ	
DP56113 SPTA Maintainer					
Leaf (V9)	41	12 - 72	16	0.27	0/32
Root (V9)	5.9	0.23 - 12	2.8	0.14	0/32
Whole Plant (V9)	27	15 - 40	6.7	0.090	0/32
Leaf (R1)	37	6.0 - 66	14	0.27	0/32
Pollen (R1)	260	110 - 380	63	0.54	0/32
Root (R1)	3.9	0.69 - 7.5	1.6	0.14	0/32
Whole Plant (R1)	17	10 - 22	2.8	0.090	0/32
Forage (R4)	5.4	2.6 - 8.0	1.3	0.090	0/32
Leaf (R4)	18	4.1 - 28	5.3	0.27	0/32
Root (R4)	1.7	0.14 - 3.9	0.99	0.14	0/32
Seed (R6)	1.3	0.60 - 2.5	0.45	0.14	0/32
Leaf (R6)	0.26[a]	<0.27 - 1.7	0.40[a]	0.27	28/32
Root (R6)	0.11[a]	<0.14 - 0.63	0.12[a]	0.14	29/32
Whole Plant (R6)	0.24[a]	<0.090 - 2.0	0.53[a]	0.090	28/32

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

[a] Some, but not all sample results were below the LLOQ. A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.

Table 12. Across-Site Summary of Expressed Trait DsRed2 Protein Concentrations of DP56113 SPTA Maintainer

Tissue (Growth Stage)	ng DsRed2/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	Range	Standard Deviation	Sample LLOQ	
DP56113 SPTA Maintainer					
Leaf (V9)	130	80 - 190	26	72	0/32
Root (V9)	40[a]	<24 - 58	12[a]	24	3/32
Whole Plant (V9)	89	60 - 120	13	36	0/32
Leaf (R1)	190	140 - 300	37	72	0/32
Pollen (R1)	<72	<72	ND	72	32/32
Root (R1)	51	31 - 85	13	24	0/32
Whole Plant (R1)	98	64 - 160	23	36	0/32
Forage (R4)	100	68 - 140	15	36	0/32
Leaf (R4)	190	78 - 320	61	72	0/32
Root (R4)	50[a]	<24 - 69	13[a]	24	1/32
Seed (R6)	480	370 - 620	59	36	0/32
Leaf (R6)	<72	<72	ND	72	32/32
Root (R6)	21[a]	<24 - 52	12[a]	24	19/32
Whole Plant (R6)	33[a]	<36 - 88	22[a]	36	21/32

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

[a] Some but not all sample results were below the LLOQ. A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.

VII. Compositional Assessment of DP56113 SPTA Maintainer Maize

An assessment of the compositional equivalence of a GE product compared to that of a conventional non-GE comparator with a history of safe use in food and feed is an important part of the weight-of-evidence approach used to evaluate the safety of genetically engineered plant products (Codex Alimentarius Commission, 2008; OECD, 1993). Compositional assessments of DP56113 SPTA maintainer were evaluated in comparison to concurrently grown non-GE, near-isoline maize (referred to as control maize) to identify statistical differences, and subsequently were evaluated in the context of normal ranges of variation established from multiple sources of non-GE, commercial maize data.

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected and analyzed for key nutritional components. The forage assessment included proximate, fiber, and mineral analytes. The seed assessment included proximate, fiber, fatty acid, amino acid, mineral, vitamin, secondary metabolite, and anti-nutrient analytes. Statistical analyses were conducted to evaluate and compare the nutrient composition of forage and grain derived from DP56113 SPTA maintainer and the control maize. If there was a statistical difference between DP56113 SPTA maintainer and the control maize for a given analyte, one or more reference ranges (*i.e.,* tolerance interval, literature range, and in-study reference range) representing the non-GE maize population with a history of safe use were utilized to evaluate statistical differences in the context of natural variation.

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

VII-A. Generation of Tissue Samples for Nutrient Composition Analysis of DP56113 SPTA Maintainer Maize

The field portion of this study was conducted during the 2017 growing season at eight sites in commercial maize-growing regions of the United States (two sites in Iowa, one site in Illinois, Indiana, Minnesota, Nebraska, Pennsylvania, and Washington). A randomized complete block design with four blocks was utilized at each site. Each block included DP56113 SPTA maintainer, control maize, and four of the following non-GM proprietary maize lines: PH12K9, PHWRW, PHB00, PHR1R, PHEJW, PH134G, PH3KP, PH1M3S, PH8JR, PH24E, PHCJP, PH1DCP, PHR62, and PHCER (referred to as reference maize).

Forage at R4 and seed at R6 growth stages were collected and analyzed for key nutritional components. Refer to Abendroth et al. (2011) for a description of maize growth stages. All

samples were collected from impartially selected, healthy, representative plants. Sample collection and processing methods are provided in Appendix 7.

VII-B. Determination of Nutrient Composition Analyte Concentrations of DP56113 SPTA Maintainer Maize

All procedures and methods for nutrient composition analyses of maize forage and grain were conducted in accordance with the requirements for the United States EPA Good Laboratory Practice (GLP) Standards, 40 CFR §160. The analytical procedures used were validated methods. The majority were based on methods published by AOAC International, AACC (American Association of Cereal Chemists), and AOCS (American Oil Chemists' Society). Details regarding the methods used for nutrient composition analysis are provided in Appendix 7.

VII-C. Assessment of Nutrient Composition of DP56113 SPTA Maintainer Maize

A total of 69 analytes were included in the statistical analysis, which included 68 original analytes, as well as one additional calculated analyte (total tocopherols, as described in Methods Appendix 7). A total of 67 analytes (9 analytes from forage, and 58 analytes from seed) were evaluated using mixed model analysis. The remaining 2 analytes did not meet criteria for sufficient quantities of observations above the LLOQ and were therefore subjected to Fisher's exact test.

No statistically significant differences were observed between DP56113 SPTA Maintainer and the control maize for 45 of the 69 analytes that went through across-site analysis via either mixed model analysis or Fisher's exact test.

Those analytes with non-significant FDR-adjusted P-values indicate that these differences were likely false positives.

For the analytes where a statistical difference was identified prior to FDR adjustment, either all sample values for DP56113 SPTA Maintainer were within the range of natural variation estimated by a tolerance interval, literature range, or in-study reference data range, with one exception (zinc in grain which had some sample values above the maximum literature range value).

VII-C.1 Proximates, Fiber, and Minerals in DP56113 SPTA Maintainer Forage

Proximates, fiber, and minerals were analyzed in forage derived from DP56113 SPTA Maintainer and control maize. Results are shown in Table 13. A statistically significant difference (P-value < 0.05) was observed between DP56113 maize and control maize mean values for ash and carbohydrates; however, all of the individual values were within the literature range, indicating DP56113 maize is within the range of normal variation for these proximates and the statistical differences are not biologically meaningful. The non-significant FDR-adjusted P-values indicate that these differences were likely false positives. Therefore, the composition of DP56113 SPTA Maintainer forage is substantially equivalent to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 13. Across-Site Analysis of Nutrient Composition Results in DP 56113 SPTA Maintainer Forage

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Proximate, Fiber, and Mineral Composition (% Dry Weight)						
Crude Protein	Mean	9.25	9.36	4.30 - 12.6	3.14 - 16.32	6.53 - 12.3
	Range	7.60 - 11.7	7.33 - 11.2			
	Confidence Interval	8.49 - 10.0	8.60 - 10.1			
	Adjusted P-Value	--	0.653			
	P-Value	--	0.497			
Crude Fat	Mean	3.90	3.88	1.04 - 5.46	ND - 6.755	2.58 - 5.27
	Range	3.05 - 4.59	2.47 - 4.87			
	Confidence Interval	3.59 - 4.20	3.57 - 4.18			
	Adjusted P-Value	--	0.926			
	P-Value	--	0.913			
Crude Fiber	Mean	24.8	25.4	14.3 - 31.0	12.5 - 42	18.3 - 28.2
	Range	20.7 - 29.6	19.9 - 28.7			
	Confidence Interval	23.6 - 25.9	24.2 - 26.5			
	Adjusted P-Value	--	0.331			
	P-Value	--	0.193			
ADF	Mean	30.9	31.4	18.7 - 39.6	9.90 - 47.39	22.9 - 35.8
	Range	25.9 - 38.1	26.1 - 38.4			
	Confidence Interval	29.3 - 32.6	29.7 - 33.0			
	Adjusted P-Value	--	0.445			
	P-Value	--	0.284			
NDF	Mean	51.7	52.2	34.0 - 62.6	20.29 - 67.80	39.0 - 56.1
	Range	45.6 - 59.2	42.4 - 59.3			
	Confidence Interval	50.0 - 53.4	50.5 - 53.9			
	Adjusted P-Value	--	0.727			
	P-Value	--	0.616			
Ash	Mean	5.51	5.60	2.66 - 10.0	0.66 - 13.20	2.85 - 12.2
	Range	2.91 - 10.2	2.93 - 8.65			
	Confidence Interval	4.31 - 6.71	4.40 - 6.80			
	Adjusted P-Value	--	0.137			
	P-Value	--	0.0492*			
Carbohydrates	Mean	81.5	81.0	76.5 - 89.5	73.3 - 92.9	72.1 - 87.4
	Range	75.5 - 85.8	77.8 - 86.2			
	Confidence Interval	79.8 - 83.2	79.3 - 82.6			
	Adjusted P-Value	--	0.0537			
	P-Value	--	0.0160*			
Calcium	Mean	0.368	0.390	0.0931 - 0.537	0.06 - 0.58	0.118 - 0.566
	Range	0.251 - 0.736	0.213 - 0.725			
	Confidence Interval	0.283 - 0.452	0.305 - 0.475			
	Adjusted P-Value	--	0.272			
	P-Value	--	0.134			
Phosphorus	Mean	0.312	0.307	0.0956 - 0.454	0.07 - 0.55	0.158 - 0.510
	Range	0.170 - 0.410	0.166 - 0.451			
	Confidence Interval	0.263 - 0.361	0.258 - 0.357			
	Adjusted P-Value	--	0.707			
	P-Value	--	0.559			

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

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

VII-C.2. Proximates and Fiber in DP56113 SPTA Maintainer Seed

Proximates and fiber were analyzed in seed derived from DP56113 SPTA Maintainer and near-isoline control. Results are shown in Table 14.

A statistical difference was identified prior to and after FDR adjustment for crude protein and carbohydrates. All sample values for carbohydrates were within the tolerance interval and literature range, and values for crude protein were within the literature range, indicating that these differences are not biologically meaningful.

The results of the analysis of proximates and fiber in maize seed demonstrate that DP56113 SPTA Maintainer is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 14. Across-Site Analysis of Proximates and Fiber Results in DP56113 SPTA Maintainer Seed

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Proximate and Fiber Composition (% Dry Weight)						
Total Dietary Fiber	Mean	9.43	9.20	5.91 - 15.8	6.68 - 35.31	6.20 - 14.2
	Range	7.21 - 12.3	6.08 - 13.0			
	Confidence Interval	8.72 - 10.1	8.49 - 9.92			
	Adjusted P-Value	--	0.727			
	P-Value	--	0.603			
Crude Protein	Mean	11.7	12.3	7.18 - 13.2	5.72 - 17.26	9.24 - 14.6
	Range	9.63 - 13.5	10.0 - 13.5			
	Confidence Interval	11.0 - 12.4	11.6 - 13.0			
	Adjusted P-Value	--	<0.0001†			
	P-Value	--	<0.0001*			
Crude Fat	Mean	4.21	4.34	2.58 - 6.00	1.363 - 7.830	3.00 - 6.15
	Range	3.64 - 4.87	3.66 - 5.31			
	Confidence Interval	4.04 - 4.39	4.17 - 4.51			
	Adjusted P-Value	--	0.440			
	P-Value	--	0.269			
Crude Fiber	Mean	2.32	2.34	1.44 - 3.48	0.49 - 5.5	1.86 - 3.16
	Range	2.01 - 2.63	1.93 - 2.80			
	Confidence Interval	2.24 - 2.39	2.27 - 2.41			
	Adjusted P-Value	--	0.755			
	P-Value	--	0.665			
ADF	Mean	4.09	4.03	2.64 - 6.26	1.41 - 11.34	2.33 - 6.36
	Range	3.38 - 4.88	2.98 - 5.77			
	Confidence Interval	3.87 - 4.31	3.81 - 4.25			
	Adjusted P-Value	--	0.727			
	P-Value	--	0.589			
NDF	Mean	11.2	11.4	7.22 - 20.8	4.28 - 22.64	8.08 - 15.2
	Range	8.85 - 14.1	9.15 - 15.1			
	Confidence Interval	10.4 - 12.0	10.7 - 12.2			
	Adjusted P-Value	--	0.594			
	P-Value	--	0.426			
Ash	Mean	1.36	1.38	0.976 - 1.80	0.616 - 6.282	1.09 - 1.75
	Range	1.24 - 1.50	1.24 - 1.51			
	Confidence Interval	1.33 - 1.39	1.35 - 1.41			
	Adjusted P-Value	--	0.319			
	P-Value	--	0.181			
Carbohydrates	Mean	82.7	82.0	80.2 - 88.0	77.4 - 89.7	78.8 - 85.5
	Range	80.9 - 84.9	80.9 - 84.5			
	Confidence Interval	82.0 - 83.4	81.3 - 82.7			
	Adjusted P-Value	--	0.0159†			
	P-Value	--	0.00237*			

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

VII-C.3. Fatty Acids in DP56113 SPTA Maintainer Seed

Fatty acids were analyzed in seed derived from DP56113 maize and near-isoline control. Results are shown in Table 15.

A statistical difference was identified prior to FDR adjustment for palmitic acid (C16:0), oleic acid (C18:1), arachidic acid (C20:0). All sample values for these analytes were within the tolerance interval indicating that the composition of DP56113 SPTA maintainer is substantially equivalent to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

A statistical difference was identified prior to and after FDR adjustment for linoleic acid (C18:2), but all values were within the literature range and therefore within the normal range of variation, indicating this statistical difference is not biologically meaningful.

The results of the analysis of fatty acids in maize seed demonstrate that DP56113 SPTA Maintainer is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 15. Across-Site Analysis of Fatty Acids Results in DP56113 SPTA Maintainer Seed

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Fatty Acid Composition (% Total Fatty Acids)						
Lauric Acid (C12:0)	Mean	<LLOQ[a]	<LLOQ[a]	0.00 - 0.209[n]	ND - 0.698	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Myristic Acid (C14:0)	Mean	<LLOQ[a]	<LLOQ[a]	0.00 - 0.267[n]	ND - 0.288	0.0356 - 0.0915
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Palmitic Acid (C16:0)	Mean	10.2	10.4	9.23 - 26.0	6.81 - 39.0	10.0 - 15.3
	Range	9.58 - 10.9	9.83 - 11.3			
	Confidence Interval	9.96 - 10.5	10.1 - 10.7			
	Adjusted P-Value	--	0.0736			
	P-Value	--	0.0231[*]			
Palmitoleic Acid (C16:1)	Mean	<LLOQ[a]	<LLOQ[a]	0 - 0.463	ND - 0.67	0.0359 - 0.178
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Heptadecanoic Acid (C17:0)	Mean	0.0778	0.0771	0 - 0.245	ND - 0.203	0.0371 - 0.154
	Range	0.0484 - 0.112	0.0444 - 0.115			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Heptadecenoic Acid (C17:1)	Mean	<LLOQ[a]	<LLOQ[a]	0.00 - 0.135[n]	ND - 0.131	0.0359 - 0.119
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Stearic Acid (C18:0)	Mean	2.17	2.14	1.31 - 3.94	ND - 4.9	1.05 - 2.72
	Range	1.74 - 2.72	1.65 - 2.70			
	Confidence Interval	1.91 - 2.46	1.89 - 2.43			
	Adjusted P-Value	--	0.758			
	P-Value	--	0.694			
Oleic Acid (C18:1)	Mean	21.3	22.0	18.9 - 39.4	16.38 - 42.81	17.3 - 40.2
	Range	17.2 - 23.0	19.1 - 23.6			
	Confidence Interval	20.1 - 22.4	20.8 - 23.1			
	Adjusted P-Value	--	0.0258[†]			
	P-Value	--	0.00616[*]			
Linoleic Acid (C18:2)	Mean	63.7	62.7	28.9 - 64.4	13.1 - 67.68	42.1 - 66.8
	Range	61.9 - 67.4	60.8 - 66.2			
	Confidence Interval	62.4 - 65.0	61.4 - 64.1			
	Adjusted P-Value	--	0.0160[†]			
	P-Value	--	0.00307[*]			
α-Linolenic Acid (C18:3)	Mean	1.43	1.43	0.0362 - 2.15	ND - 2.33	0.788 - 2.07
	Range	1.15 - 1.65	1.18 - 1.65			
	Confidence Interval	1.32 - 1.54	1.31 - 1.54			
	Adjusted P-Value	--	0.795			
	P-Value	--	0.771			
Arachidic Acid (C20:0)	Mean	0.342	0.357	0.296 - 0.916	0.267 - 1.2	0.255 - 0.488
	Range	0.298 - 0.380	0.309 - 0.408			
	Confidence Interval	0.322 - 0.362	0.337 - 0.377			
	Adjusted P-Value	--	0.0863			
	P-Value	--	0.0284[*]			

Table 15. Across-Site Analysis of Fatty Acids Results in DP56113 SPTA Maintainer Seed (continued)

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Fatty Acid Composition (% Total Fatty Acids)						
Eicosenoic Acid (C20:1)	Mean	0.247	0.251			
	Range	0.216 - 0.327	0.208 - 0.366			
	Confidence Interval	0.232 - 0.263	0.236 - 0.267	0.0380 - 0.693	ND - 1.952	0.183 - 0.486
	Adjusted P-Value	--	0.727			
	P-Value	--	0.618			
Eicosadienoic Acid (C20:2)	Mean	<LLOQ[a]	<LLOQ[a]			
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA	0.00 - 0.825[◦]	ND - 2.551	0.0361 - 0.107
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Behenic Acid (C22:0)	Mean	0.108	0.112			
	Range	0.0844 - 0.190	0.0872 - 0.227			
	Confidence Interval	NA	NA	0 - 0.453	ND - 0.5	0.0816 - 0.277
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Lignoceric Acid (C24:0)	Mean	0.196	0.209			
	Range	0.102 - 0.290	0.102 - 0.301			
	Confidence Interval	0.145 - 0.247	0.158 - 0.260	0 - 0.639	ND - 0.91	0.104 - 0.392
	Adjusted P-Value	--	0.189			
	P-Value	--	0.0733			

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

[a] < LLOQ; all fatty acid sample values were below the assay LLOQ on the fresh weight basis, and no LLOQ value was determined on the % total fatty acids basis.

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

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

[†] Adjusted P-Value < 0.05 was observed.

VII-C.4 Amino Acids in DP56113 SPTA Maintainer Seed

Amino acids were analyzed in seed derived from DP56113 SPTA Maintainer and near-isoline control. Results are shown in Table 16.

A statistical difference was identified for lysine prior to FDR adjustment, but has a non-significant FDR-adjusted P-value, which indicates that this difference is likely a false positive.

A statistical difference was identified prior to and after FDR adjustment for alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, proline, serine, threonine, and valine but all values were within the tolerance interval, indicating these statistical differences are not biologically meaningful.

The results of the analysis of amino acids in maize seed demonstrate that DP56113 SPTA Maintainer is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 16. Across-Site Analysis of Amino Acids Results in DP56113 SPTA Maintainer Seed

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Amino Acid Composition (% Dry Weight)						
Alanine	Mean	0.906	0.952	0.492 - 1.08	0.44 - 1.48	0.620 - 1.17
	Range	0.726 - 1.11	0.767 - 1.08			
	Confidence Interval	0.845 - 0.967	0.891 - 1.01			
	Adjusted P-Value	--	0.00124†			
	P-Value	--	<0.0001*			
Arginine	Mean	0.456	0.462	0.317 - 0.568	0.12 - 0.71	0.342 - 0.569
	Range	0.390 - 0.524	0.403 - 0.518			
	Confidence Interval	0.432 - 0.481	0.438 - 0.486			
	Adjusted P-Value	--	0.440			
	P-Value	--	0.266			
Aspartic Acid	Mean	0.715	0.757	0.445 - 0.916	0.33 - 1.21	0.576 - 0.921
	Range	0.564 - 0.862	0.620 - 0.851			
	Confidence Interval	0.671 - 0.760	0.713 - 0.802			
	Adjusted P-Value	--	0.000720†			
	P-Value	--	<0.0001*			
Cystine	Mean	0.216	0.218	0.132 - 0.303	0.12 - 0.51	0.0745 - 0.285
	Range	0.0979 - 0.267	0.100 - 0.268			
	Confidence Interval	0.196 - 0.233	0.198 - 0.234			
	Adjusted P-Value	--	0.795			
	P-Value	--	0.760			
Glutamic Acid	Mean	2.34	2.45	1.04 - 2.70	0.97 - 3.54	1.63 - 3.07
	Range	1.82 - 2.84	1.92 - 2.92			
	Confidence Interval	2.17 - 2.52	2.28 - 2.63			
	Adjusted P-Value	--	0.0396†			
	P-Value	--	0.0112*			
Glycine	Mean	0.410	0.421	0.292 - 0.487	0.184 - 0.685	0.285 - 0.500
	Range	0.356 - 0.460	0.368 - 0.486			
	Confidence Interval	0.388 - 0.432	0.399 - 0.443			
	Adjusted P-Value	--	0.0351†			
	P-Value	--	0.00944*			
Histidine	Mean	0.316	0.322	0.177 - 0.359	0.14 - 0.46	0.238 - 0.396
	Range	0.270 - 0.354	0.289 - 0.366			
	Confidence Interval	0.303 - 0.329	0.309 - 0.335			
	Adjusted P-Value	--	0.212			
	P-Value	--	0.0853			
Isoleucine	Mean	0.425	0.447	0.229 - 0.494	0.18 - 0.69	0.298 - 0.513
	Range	0.344 - 0.500	0.364 - 0.511			
	Confidence Interval	0.395 - 0.454	0.417 - 0.476			
	Adjusted P-Value	--	0.0160†			
	P-Value	--	0.00335*			
Leucine	Mean	1.53	1.61	0.763 - 1.85	0.64 - 2.49	0.997 - 1.88
	Range	1.16 - 1.87	1.23 - 1.87			
	Confidence Interval	1.40 - 1.66	1.48 - 1.74			
	Adjusted P-Value	--	0.0160†			
	P-Value	--	0.00301*			
Lysine	Mean	0.308	0.327	0.186 - 0.412	0.129 - 0.668	0.222 - 0.398
	Range	0.241 - 0.356	0.262 - 0.403			
	Confidence Interval	0.288 - 0.328	0.307 - 0.347			
	Adjusted P-Value	--	0.123			
	P-Value	--	0.0421*			
Methionine	Mean	0.311	0.322	0.108 - 0.342	0.10 - 0.47	0.0788 - 0.352
	Range	0.144 - 0.407	0.150 - 0.407			
	Confidence Interval	0.276 - 0.340	0.290 - 0.349			
	Adjusted P-Value	--	0.244			
	P-Value	--	0.113			

Table 16. Across-Site Analysis of Amino Acids Results in DP56113 SPTA Maintainer Seed (continued)

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Amino Acid Composition (% Dry Weight)						
Phenylalanine	Mean	0.631	0.665	0.342 - 0.736	0.24 - 0.93	0.434 - 0.762
	Range	0.497 - 0.741	0.532 - 0.787			
	Confidence Interval	0.579 - 0.684	0.612 - 0.718			
	Adjusted P-Value	--	0.0313†			
	P-Value	--	0.00793*			
Proline	Mean	1.08	1.12	0.597 - 1.25	0.46 - 1.75	0.748 - 1.28
	Range	0.883 - 1.24	0.914 - 1.26			
	Confidence Interval	1.02 - 1.14	1.06 - 1.18			
	Adjusted P-Value	--	0.00666†			
	P-Value	--	0.000796*			
Serine	Mean	0.595	0.618	0.296 - 0.677	0.18 - 0.91	0.422 - 0.720
	Range	0.487 - 0.691	0.519 - 0.706			
	Confidence Interval	0.562 - 0.629	0.585 - 0.652			
	Adjusted P-Value	--	0.00464†			
	P-Value	--	0.000415*			
Threonine	Mean	0.421	0.439	0.179 - 0.476	0.22 - 0.67	0.305 - 0.496
	Range	0.358 - 0.472	0.376 - 0.490			
	Confidence Interval	0.400 - 0.442	0.418 - 0.459			
	Adjusted P-Value	--	0.00124†			
	P-Value	--	<0.0001*			
Tryptophan	Mean	0.0701	0.0715	0.0405 - 0.0913	0.027 - 0.215	0.0475 - 0.0909
	Range	0.0615 - 0.0799	0.0653 - 0.0782			
	Confidence Interval	0.0674 - 0.0727	0.0688 - 0.0742			
	Adjusted P-Value	--	0.272			
	P-Value	--	0.131			
Tyrosine	Mean	0.359	0.370	0.164 - 0.421	0.10 - 0.73	0.228 - 0.443
	Range	0.299 - 0.455	0.278 - 0.442			
	Confidence Interval	0.333 - 0.385	0.344 - 0.396			
	Adjusted P-Value	--	0.236			
	P-Value	--	0.106			
Valine	Mean	0.514	0.540	0.318 - 0.626	0.21 - 0.86	0.376 - 0.660
	Range	0.436 - 0.586	0.463 - 0.600			
	Confidence Interval	0.488 - 0.540	0.514 - 0.566			
	Adjusted P-Value	--	0.0177†			
	P-Value	--	0.00397*			

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

VII-C.5 Minerals in DP56113 SPTA Maintainer Seed

Minerals were analyzed in seed derived from DP56113 SPTA Maintainer and near-isoline control. Results are shown in Table 17.

A statistical difference (P-value <0.05) was identified prior to and after FDR adjustment for copper and zinc. All values for copper were within the tolerance interval, literature range and within normal range of variation, indicating this statistical difference is not biologically meaningful.

Results for zinc values in seed had 7 out 32 individual sample values for DP56113 maize above the maximum literature range value. The magnitude of difference between the means of DP56113 maize and the near-isoline control was small (+6%). Maize is not a major food source in the human diet (NIH-ODS, 2019), and maize grain concentrations of minerals are typically not a concern for animal diets with the common addition of mineral premixes to formulations (NRC, 1994; NRC, 1996; NRC, 1998), therefore the small difference observed do not have biological relevance from a food and feed safety perspective.

The results of the analysis of minerals in maize seed demonstrate that DP56113 SPTA Maintainer is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize, with the exception of zinc.

Table 17. Across-Site Analysis of Minerals Results in DP56113 SPTA Maintainer Seed

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Mineral Composition (% Dry Weight)						
Calcium	Mean	0.00333	0.00345	0.00131 - 0.00784	ND - 0.101	0.00153 - 0.00833
	Range	0.00211 - 0.00683	0.00241 - 0.00440			
	Confidence Interval	0.00291 - 0.00376	0.00302 - 0.00388			
	Adjusted P-Value	--	0.308			
	P-Value	--	0.165			
Copper	Mean	0.0000838	0.0000687	<0.0000625 - 0.000617	ND - 0.0021	<0.0000625[b] - 0.000324
	Range	<0.0000625[b] - 0.000125	<0.0000625[b] - 0.000120			
	Confidence Interval	0.0000602 - 0.000107	0.0000450 - 0.0000924			
	Adjusted P-Value	--	0.00564[†]			
	P-Value	--	0.000590[*]			
Iron	Mean	0.00226	0.00239	0.00118 - 0.00261	0.0000712 - 0.0191	0.00133 - 0.00593
	Range	0.00169 - 0.00270	0.00183 - 0.00266			
	Confidence Interval	0.00215 - 0.00237	0.00228 - 0.00250			
	Adjusted P-Value	--	0.164			
	P-Value	--	0.0613			
Magnesium	Mean	0.125	0.127	0.0787 - 0.163	0.0035 - 1.000	0.0879 - 0.152
	Range	0.114 - 0.141	0.113 - 0.137			
	Confidence Interval	0.122 - 0.129	0.123 - 0.130			
	Adjusted P-Value	--	0.598			
	P-Value	--	0.437			
Manganese	Mean	0.000571	0.000551	0.000328 - 0.00131	0.0000312 - 0.0054	0.000366 - 0.00121
	Range	0.000388 - 0.000709	0.000384 - 0.000677			
	Confidence Interval	0.000519 - 0.000623	0.000499 - 0.000603			
	Adjusted P-Value	--	0.229			
	P-Value	--	0.0967			
Phosphorus	Mean	0.335	0.342	0.204 - 0.429	0.010 - 0.750	0.215 - 0.419
	Range	0.277 - 0.399	0.283 - 0.391			
	Confidence Interval	0.319 - 0.351	0.326 - 0.358			
	Adjusted P-Value	--	0.229			
	P-Value	--	0.0990			
Potassium	Mean	0.295	0.300	0.222 - 0.541	0.18 - 0.720	0.282 - 0.466
	Range	0.264 - 0.336	0.257 - 0.342			
	Confidence Interval	0.284 - 0.306	0.289 - 0.311			
	Adjusted P-Value	--	0.482			
	P-Value	--	0.324			
Sodium	Mean	0.000150	0.000192	0.00000298 - 0.00366	ND - 0.150	<0.0000625[b] - 0.000862
	Range	<0.0000625[b] - 0.000758	<0.0000625[b] - 0.000658			
	Confidence Interval	0.0000973 - 0.000202	0.000139 - 0.000244			
	Adjusted P-Value	--	0.275			
	P-Value	--	0.140			
Zinc	Mean	0.00354	0.00376	0.00140 - 0.00365	0.0000283 - 0.0043	0.00189 - 0.00384
	Range	0.00264 - 0.00512	0.00281 - 0.00519			
	Confidence Interval	0.00305 - 0.00404	0.00327 - 0.00426			
	Adjusted P-Value	--	0.00308[†]			
	P-Value	--	0.000229[*]			

Note: Not detectable (ND); one or more assay values in the published literature references were below the LLOQ and were not quantified.

[b] < LLOQ; (where a numerical number for LLOQ value is reported, *e.g.* < 0.0000625 for Sodium), one or more mineral sample values were below the assay LLOQ.

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

[†] Adjusted P-Value < 0.05 was observed.

VII-C.6 Vitamins in DP56113 SPTA Maintainer Seed

Vitamins were analyzed in seed derived from DP56113 maize and near-isoline control. Results are shown in Table 18.

A statistically significant difference (P-value < 0.05) was observed between DP56113 SPTA Maintainer and control maize mean values for vitamin B1 (thiamine) and vitamin B5 (pantothenic acid); however, all the individual values were within the tolerance interval for vitamin B1 and within the literature range for vitamin B5, indicating DP56113 SPTA Maintainer is within the range of normal variation for these vitamins and the statistical differences are not biologically meaningful. The non-significant FDR-adjusted P-value indicate that the difference for vitamin B1 was likely false a positive.

The results of the analysis of vitamins in maize seed demonstrate that DP56113 SPTA Maintainer is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 18. Across-Site Analysis of Vitamins Results in DP56113 SPTA Maintainer Seed

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Vitamin Composition (mg/kg Dry Weight)						
β-Carotene	Mean	0.835	0.808	<0.0500 - 2.06[a]	0.3 - 5.4	0.255 - 1.86
	Range	0.318 - 1.38	0.345 - 1.33			
	Confidence Interval	0.618 - 1.05	0.591 - 1.03			
	Adjusted P-Value	--	0.519			
	P-Value	--	0.364			
Vitamin B1 (Thiamine)	Mean	2.85	3.03	1.71 - 5.38	ND - 40.00	1.78 - 4.45
	Range	2.23 - 3.56	2.28 - 3.89			
	Confidence Interval	2.58 - 3.11	2.77 - 3.29			
	Adjusted P-Value	--	0.00836[†]			
	P-Value	--	0.00112[*]			
Vitamin B2 (Riboflavin)	Mean	<0.900[b]	<0.900[b]	<0.900 - 2.27[a]	ND - 7.35	<0.900[b]
	Range	<0.900[b]	<0.900[b]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Vitamin B3 (Niacin)	Mean	15.3	15.5	7.86 - 25.2	ND - 70	8.52 - 29.8
	Range	12.1 - 22.1	12.0 - 25.1			
	Confidence Interval	14.0 - 16.6	14.3 - 16.8			
	Adjusted P-Value	--	0.691			
	P-Value	--	0.537			
Vitamin B5 (Pantothenic Acid)	Mean	4.96	4.78	3.05 - 7.66	3.0 - 14	<2.67[b] - 7.01
	Range	2.76 - 6.12	3.01 - 6.03			
	Confidence Interval	4.32 - 5.60	4.15 - 5.42			
	Adjusted P-Value	--	0.0160[†]			
	P-Value	--	0.00323[*]			
Vitamin B6 (Pyridoxine)	Mean	2.34	2.38	1.37 - 8.67	ND - 12.14	1.45 - 7.63
	Range	1.12 - 3.66	1.17 - 4.17			
	Confidence Interval	1.79 - 2.88	1.84 - 2.93			
	Adjusted P-Value	--	0.795			
	P-Value	--	0.751			
Vitamin B9 (Folic Acid)	Mean	1.15	1.15	0.319 - 2.41	ND - 3.50	0.348 - 2.70
	Range	0.0374 - 1.85	0.468 - 2.35			
	Confidence Interval	0.862 - 1.44	0.862 - 1.44			
	Adjusted P-Value	--	0.999			
	P-Value	--	0.999			
α-Tocopherol	Mean	3.69	3.81	0 - 25.1	ND - 68.67	<0.500[b] - 25.8
	Range	<0.500[b] - 6.07	1.51 - 7.54			
	Confidence Interval	2.81 - 4.57	2.93 - 4.69			
	Adjusted P-Value	--	0.758			
	P-Value	--	0.684			
β-Tocopherol	Mean	<0.500[b]	<0.500[b]	<0.500 - 1.10[a]	ND - 19.80	<0.500[b] - 1.90
	Range	<0.500[b]	<0.500[b]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
γ-Tocopherol	Mean	21.3	20.5	0 - 46.5	ND - 58.61	11.7 - 56.8
	Range	15.6 - 27.8	13.5 - 25.3			
	Confidence Interval	19.5 - 23.2	18.6 - 22.3			
	Adjusted P-Value	--	0.319			
	P-Value	--	0.179			

Table 18. Across-Site Analysis of Vitamins Results in DP56113 SPTA Maintainer Seed (continued)

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Vitamin Composition (mg/kg Dry Weight)						
δ-Tocopherol	Mean	<0.500[b]	<0.500[b]			
	Range	<0.500[b]	<0.500[b]			
	Confidence Interval	NA	NA	<0.500 - 2.61[▫]	ND - 14.61	<0.500[b] - 3.45
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Total Tocopherols	Mean	25.5	24.8			
	Range	19.1 - 32.1	17.6 - 30.5			
	Confidence Interval	23.1 - 28.0	22.3 - 27.2	0 - 61.0	ND - 89.91	20.5 - 62.0
	Adjusted P-Value	--	0.465			
	P-Value	--	0.305			

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

[b] < LLOQ; (where a numerical number for LLOQ value is reported, *e.g.* < 0.900 for Vitamin B2 (Riboflavin)), one or more vitamin sample values were below the assay LLOQ.

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

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

[†] Adjusted P-Value < 0.05 was observed.

VII-C.7 Secondary Metabolites and Anti-Nutrients in DP56113 SPTA Maintainer Seed

Secondary metabolites and anti-nutrients were analyzed in seed derived from DP56113 maize and near-isoline control. Results are shown in Table 19. No statistically significant differences (P-value < 0.05) were observed between DP56113 SPTA Maintainer and control maize.

The results of the analysis of secondary metabolites and anti-nutrients in maize seed demonstrate that DP56113 SPTA Maintainer is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 19. Across-Site Analysis of Secondary Metabolite and Anti-Nutrient Results in DP56113 SPTA Maintainer Seed

Analyte	Reported Statistics	Control Maize	DP56113 Maize	Tolerance Interval	Literature Range	Reference Data Range
Secondary Metabolite and Anti-Nutrient Composition (% Dry Weight or as Indicated)						
p-Coumaric Acid	Mean	0.0308	0.0312	0.00715 - 0.0521	ND - 0.08	0.00702 - 0.0492
	Range	0.0199 - 0.0412	0.0105 - 0.0362			
	Confidence Interval	0.0293 - 0.0324	0.0297 - 0.0328			
	Adjusted P-Value	--	0.758			
	P-Value	--	0.701			
Ferulic Acid	Mean	0.238	0.239	0.109 - 0.359	0.02 - 0.44	<0.0300[b] - 0.363
	Range	0.148 - 0.300	0.0626 - 0.282			
	Confidence Interval	0.222 - 0.254	0.223 - 0.255			
	Adjusted P-Value	--	0.747			
	P-Value	--	0.647			
Furfural	Mean	<0.000100[b]	<0.000100[b]	<0.000100	ND	<0.000100[b]
	Range	<0.000100[b]	<0.000100[b]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Inositol	Mean	0.0213	0.0222	0.00684 - 0.0509	0.0063 - 0.48	0.0118 - 0.0447
	Range	0.0130 - 0.0303	0.0135 - 0.0327			
	Confidence Interval	0.0179 - 0.0246	0.0189 - 0.0256			
	Adjusted P-Value	--	0.308			
	P-Value	--	0.165			
Phytic Acid	Mean	1.07	1.11	0.516 - 1.37	ND - 1.940	0.643 - 1.54
	Range	0.661 - 1.34	0.689 - 1.46			
	Confidence Interval	0.975 - 1.16	1.01 - 1.20			
	Adjusted P-Value	--	0.641			
	P-Value	--	0.478			
Raffinose	Mean	0.149	0.139	0 - 0.440	ND - 0.466	<0.0800[b] - 0.458
	Range	<0.0800[b] - 0.310	<0.0800[b] - 0.287			
	Confidence Interval	0.0757 - 0.222	0.0661 - 0.213			
	Adjusted P-Value	--	0.445			
	P-Value	--	0.286			
Trypsin Inhibitor (TIU/mg DW)	Mean	2.64	2.56	1.02 - 5.68	ND - 8.42	1.11 - 3.52
	Range	1.85 - 3.45	1.75 - 3.44			
	Confidence Interval	2.41 - 2.88	2.32 - 2.79			
	Adjusted P-Value	--	0.490			
	P-Value	--	0.336			

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

[b] < LLOQ; (where a numerical number for LLOQ value is reported, *e.g.* < 0.000100 for Furfural), one or more secondary metabolite or anti-nutrient sample values were below the assay LLOQ.

VII-D. Conclusions on Compositional Assessment of DP56113 SPTA Maintainer

A compositional comparative assessment was conducted to determine if DP56113 SPTA Maintainer seed and forage would present any new or greater risks relative to conventional maize varieties that have a history of safe use as food and feed. An appropriate comparator was used to determine if DP56113 SPTA Maintainer is comparable to conventional maize.

The compositional analyses of seed included crude protein, crude fat, crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, fatty acids, total amino acids, key anti-nutrients, and key secondary metabolites. Compositional analyses of forage included crude protein, crude fat, crude fiber, ADF, NDF, ash, carbohydrates, calcium, and phosphorus. In total, data from 69 different analytical components (60 in seed, nine in forage) were presented and discussed. Statistical differences were observed in 45 of the 69 analytes measured between DP56113 maize and its near-isoline control. All but one (zinc in grain) of the statistical differences were not significant when FDR was applied, were within the tolerance interval, or within the literature range. The difference in zinc was determined to not be biologically relevant from a food and feed safety perspective. Based on these analyses, the seed and forage of DP56113 SPTA maintainer are comparable to conventional maize with respect to nutrient composition.

Based on the results of the compositional evaluation, the seed and forage of DP56113 SPTA maintainer are as safe as commodity maize and is not expected to result in any significant impact on raw or processed maize commodities.

VIII. Agronomic Performance and Ecological Observations of SPTA Maintainer

Agronomic and ecological evaluations were conducted to assess the comparability of DP56113 SPTA maintainer to non-GE conventional maize. These evaluations form the basis to determine whether DP56113 SPTA maintainer is comparable to conventional maize and is therefore no more likely to pose a plant pest risk.

Agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials. To evaluate the agronomic characteristics of DP56113 SPTA maintainer, data were collected on representative characteristics that influence reproduction, crop survival, and potential weediness. In each of these assessments, DP56113 SPTA maintainer was compared to a control maize line that was >95% genetically similar to DP56113 SPTA maintainer but did not carry any recombinant DNA, and, in some experiments, was compared to non-GE conventional maize lines selected from current Pioneer conventional maize products. In each experiment, DP56113 SPTA maintainer was comparable to the control or conventional comparators.

The ecological evaluations included observed responses to insect and disease stressors during multi-site field trials. These observations were made on DP56113 SPTA maintainer and control maize and tracked the presence of insect and disease stressors in the field and the plant responses. In each case, DP56113 SPTA maintainer responded similarly to the control plants in these trials.

Based on the analyses described below, DP56113 SPTA maintainer is comparable to conventional maize and similar to the antecedent 32138 SPT maintainer. DP56113 SPTA maintainer is not anticipated to pose a greater plant pest risk or increased weed potential than conventional maize.

VIII-A. Germination and Viability Evaluations

In order to evaluate germination and dormancy, seeds from the F1 generation (Figure 2 and Table 3) of DP56113 SPTA maintainer were tested for germination assays under warm, cold, and diurnal conditions (Table 13). The F1 generation of seed was used because hybrid seed is representative of seed that growers would plant in commercial maize fields. A non-GE, near-isoline control was used for comparison. In addition, six non-GE conventionally bred maize lines, (PH24E, PHB00, PH3KP, PHCER, PHCJP, and PHEJW), were evaluated in the study to establish a reference range for germination and dormancy evaluations but were not included in the statistical analysis. This reference range provided context for any statistical differences observed in the comparisons; if the values for DP56113 SPTA maintainer fell within this reference range, it indicated that DP56113 SPTA maintainer maize was comparable to non-GE conventionally bred lines.

Each germination test contained eight replicates of 50 seeds each of DP56113 SPTA maintainer, control, and six conventional lines. The "International Rules for Seed Testing 2017", published by the International Seed Testing Association, were used as guidelines for the germination methods and interpretation of results (ISTA, 2017). Each replicate was placed between sheets of moist germination paper and rolled up with a piece of wax paper wrapped around the moist paper, and placed in a growth chamber set to the appropriate test conditions as specified in Table 20. Evaluations were taken at the end of each germination test, and the number of normal and abnormal germinated seed as well as the number of hard, fresh, or dead ungerminated seed in each roll were counted. Descriptions of germination test classifications are provided in Table 21. Germination rates were reported as a percentage of germinating seed as follows: (number of germinated seeds/total seeds planted) *100. The results are presented in Tables 22, 23, and 24.

For evaluation of viability, germinated seed were considered viable and ungerminated seed classified as dead were considered non-viable. Ungerminated seed classified as hard or fresh were further evaluated using a tetrazolium chloride (TZ) test to assess viability.

The data obtained in this study demonstrated that germination rates and viability of seed from DP56113 SPTA maintainer under cold growing conditions were comparable to those of the control maize and non-GE conventionally bred maize under corresponding growing conditions. A statistically significant difference for both warm and diurnal germination tests between DP56113 SPTA maintainer and control maize was demonstrated, but the mean germination rates for DP56113 SPTA maintainer in both the warm and diurnal germination tests exceeded recognized commercial standards for maize seed.

The data provided here support the conclusion that DP56113 SPTA maintainer is comparable to conventional maize with respect to germination and viability.

Table 20. Description of Seed Germination Conditions

Warm Germination Test	• Continuous setting of 25°C and 90% relative humidity for 7 days • Evaluated after 7 days
Cold Germination Test	• Continuous setting of 10 °C and 90% relative humidity for 7 days, followed by 5 days at a continuous setting of 25 °C and 90% relative humidity • Evaluated after 12 days
Diurnal Germination Test	• Cyclical setting of 10 °C and 90% relative humidity for 16 hours and then 25 °C and 90% relative humidity for 8 hours, repeated daily for 10 days • Evaluated after 10 days

Table 21. Description of Germination Test Classifications

Germination Classification		**Description**
Germinated Seed	Normal	Show the potential for continued development into satisfactory plants when grown in favorable conditions. All essential structures are well developed, complete, and healthy.
	Abnormal	Do not show the potential to develop into a normal plant when growing in favorable conditions. Essential structures may be damaged, deformed, or decayed to the extent that normal plant development is/ or is expected to be prevented.
Ungerminated Seed	Hard	No emergence of essential structures. Seed which remain hard at the end of the test period, because they have not absorbed water.
	Fresh	No emergence of essential structures. Seed, other than hard seed, which have failed to germinate under the conditions of the germination test, but which remain clean and firm and have the potential to develop into a normal seedling.
	Dead	No emergence of essential structures. Seed, which at the end of the test period, are neither hard nor fresh, nor have produced any part of a seedling.

Note: Germination test classifications (ISTA, 2017).

Table 22. Summary of DP56113 SPTA Maintainer Warm Germination Test Results

Reported Statistics	DP56113 SPT Maintainer	Control Maize	Reference Range
Frequency[a]	378/400	399/400	94.0% - 100%
Mean (SE)[b]	94.5% (1.14%)	99.7% (0.250%)	
Range[c]	90.0% - 100%	98.0% - 100%	
P-Value[d]	0.0022[e]	--	

[a] Total germination frequency across replicates.
[b] Mean and the standard error (SE) of the mean generated by a generalized linear mixed model (GLMM).
[c] Range of germination rates for individual replicates.
[d] P-Value was determined using GLMM-based statistical test for mean germination rates.
[e] A statistically significant difference (P-Value <0.05) was observed.

Table 23. Summary of DP56113 SPTA Maintainer Cold Germination Test Results

Reported Statistics	DP56113 SPT Maintainer	Control Maize	Reference Range
Frequency[a]	389/400	396/400	86.0% - 100%
Mean (SE)[b]	97.3% (0.818%)	99.0% (0.497%)	
Range[c]	96.0% - 100%	98.0% - 100%	
P-Value[d]	0.0801	--	

[a] Total germination frequency across replicates.
[b] Mean and the standard error (SE) of mean generated by a generalized linear mixed model (GLMM).
[c] Range of germination rates for individual replicates.
[d] P-Value was determined using GLMM-based statistical test for mean germination rates.

Table 24. Summary of DP56113 SPTA Maintainer Diurnal Germination Test Results

Reported Statistics	DP56113 SPT Maintainer	Control Maize	Reference Range
Frequency[a]	381/400	399/400	92.0% - 100%
Mean (SE)[b]	95.6% (1.29%)	99.8% (0.232%)	
Range[c]	86.0% - 98.0%	98.0% - 100%	
P-Value[d]	0.0045[e]	--	

[a] Total germination frequency across replicates.
[b] Mean and the standard error (SE) of mean generated by a generalized linear mixed model (GLMM).
[c] Range of germination rates for individual replicates.
[d] P-Value was determined using GLMM-based statistical test for mean germination rates.
[e] A statistically significant difference (P-Value <0.05) was observed.

Table 25. Tetrazolium Chloride (TZ) Testing of DP56113 SPTA Maintainer Ungerminated Seed

Crop	Germination Test	Identity Description	Number of TZ Tested Seed	TZ Test Results	
				Fresh (Viable)	Dead (Non-Viable)
Maize	Warm	PHCJP	5	1	4
		PHEJW	6	1	5
	Cold	PHCJP	34	3	31
	Diurnal	PH24E	15	2	13

VIII-B. Field Trial Evaluations of DP56113 SPTA Maintainer

Agronomic characteristic data were collected from the T4 generation of DP56113 SPTA maintainer and control maize during the 2017 growing season at eight sites in commercial maize-growing regions of the United States (two sites in Iowa, one site in Illinois, Indiana, Minnesota, Nebraska, Pennsylvania, and Washington). The trial locations provided a range of environmental and agronomic conditions representative of the major maize growing regions of the United States (Figure 21). Agronomic characteristics observed are provided in Table 26.

Agronomic characteristics of DP56113 SPTA maintainer were evaluated in comparison to concurrently grown control maize to identify statistical differences, and subsequently were evaluated in the context of normal ranges of variation established from concurrently grown non-GE, conventionally bred maize (referred to as reference maize) data.

Evaluation of agronomic characteristics of DP56113 SPTA maintainer included the following agronomic endpoints: early stand count, time to flowering, plant height, lodging, final stand count, days to maturity, pollen viability, ear count, harvest grain moisture, yield, and 100-kernel weight. Additionally, biotic and abiotic observations were taken by evaluating insect damage incidence, plant pathogen incidence, and abiotic stress at each site throughout the growing season. Statistical analysis was conducted to evaluate and compare agronomic results derived from DP56113 maize to the control maize. Biotic and abiotic stress were evaluated by recording the severity of plant tissue damage caused by each of three insects predominant to the local area, three pathogens predominant to the local area, and three abiotic stressors, respectively.

Each field trial site was managed to maintain an environment that would produce a successful crop including insect, weed, fertility and irrigation management as needed. Maintenance practices were uniform across all entries in each site, thus agronomic characteristic evaluations comparing DP56113 SPTA maintainer to control maize are appropriate.

A randomized complete block design with four blocks was utilized at each site. Each block included DP56113 maize, non-genetically modified (non-GE) near-isoline control maize (referred to as control maize), and four of the following non-GE proprietary maize lines: PH12K9, PHWRW, PHB00, PHR1R, PHEJW, PH134G, PH3KP, PH1M3S, PH8JR, PH24E, PHCJP, PH1DCP, PHR62, and PHCER (referred to as reference maize). These reference maize lines were chosen to represent a range of non-genetically engineered maize lines that are planted commercially. Agronomic data collected from the reference maize were used to help determine the normal range of variation for the agronomic characteristics in conventionally bred, non-GE maize.

Results are presented in Table 27. Details of the methods used are presented in Appendix 8.

Figure 21. Distribution of Field Locations – 2017 DP56113 SPTA Maintainer Field Trials

A total of 18 agronomic endpoints were included in the assessment: Twelve were evaluated using mixed model analysis, four agronomic characteristics were evaluated using the generalized Cochran-Mantel-Haenszel (CMH) test. The remaining two agronomic characteristics did not meet criteria for minimum levels of non-uniformity and were therefore not subjected to comparative analyses (pollen viability-shape at 120 minutes and pollen viability color at 120 minutes).

No statistically significant differences were identified between DP56113 SPTA maintainer and the control maize for 14 agronomic characteristics that went through across-site analysis.

A statistically significant difference, before and after the FDR-adjustment, between DP56113 SPTA maintainer and the control maize was observed in the across-site analysis for yield and 100-kernel weight.

For yield, all values for DP56113 SPTA maintainer were within the reference data range. A statistical difference was observed in the individual-site analyses at five sites.

For 100-kernel weight, all values for DP56113 SPTA maintainer were within the reference data range. A statistical difference was observed in the individual-site analyses at two sites.

Table 26. Agronomic Characteristics Measured

Characteristic	Evaluation Timing[a]	Description	Scale
Early Stand Count	V2-V4	Total number of plants emerged per plot divided by area to calculate plants per m^2	Numerical count per meter squared
Time to Flowering	50% of plants shedding pollen	Calculated heat units from the time of planting until approximately 50% of plants have tassels shedding pollen	Heat Units
Plant Height	R4	Average height of five plants from soil surface to collar of flag leaf	Centimeters
Lodging	R6	Combined score of stalk lodging (number of plants in each plot with stalks broken below the primary ear) and root lodging (number of plants in each plot with stalks leaning approximately 45 degrees or more)	Percentage
Final Stand Count	R6	Total number of remaining plants per plot divided by area to calculate plants per m^2	Count per meter squared
Days to Maturity	Physiological maturity	Number of days for 90% of plants to reach physiological maturity	Days
Pollen Viability[b]	During active pollen shed	Shape and color at 0, 30, 60, and 120 minutes	Percent of seeds with collapsed walls and yellow color
Ear Count	R6	Average number of ears per plant of five plants.	Count
Harvest Seed Moisture	R6	Moisture content of harvested seed	Percent
Yield	R6	Harvest weight per area adjusted to 15.5% moisture	Calculated bushels per acre
100 Kernel Weight	R6	Total weight of 100 kernels of pooled seed, adjusted for moisture	Weight in grams

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

Table 27. Across-Site Analysis of DP56113 SPTA Maintainer Agronomic Characteristics

Agronomic Characteristic	Reported Statistics	Control Maize	DP56113 SPTA Maintainer	Reference Data Range
Early Stand (count/m^2)	Mean	5.6	5.4	
	Range	4.1 - 6.2	4.5 - 6.0	
	Confidence Interval	5.2 - 6.0	5.0 - 5.8	1.7 - 6.4
	Adjusted P-Value	--	0.425	
	P-Value	--	0.116	
Time to Flowering (heat units)	Mean	1440	1440	
	Range	1300 - 1520	1320 - 1560	
	Confidence Interval	1390 - 1490	1390 - 1490	1250 - 1570
	Adjusted P-Value	--	0.425	
	P-Value	--	0.133	
Pollen Viability-Shape, 0 minutes (% of pollen with collapsed walls)	Mean	12.3	10.3	
	Range	0 - 50	0 - 30	
	Confidence Interval	5.4 - 19.1	3.5 - 17.2	0 - 60
	Adjusted P-Value	--	0.534	
	P-Value	--	0.213	
Pollen Viability-Shape, 30 minutes (% of pollen with collapsed walls)	Mean	67.8	63.0	
	Range	20 - 100	20 - 100	
	Confidence Interval	45.2 - 90.4	40.4 - 85.5	5 - 100
	Adjusted P-Value	--	0.534	
	P-Value	--	0.357	
Pollen Viability-Shape, 60 minutes (% of pollen with collapsed walls)	Mean	91.1	88.1	
	Range	60 - 100	50 - 100	
	Confidence Interval	NA	NA	50 - 100
	Adjusted P-Value	--	0.425	
	P-Value	--	0.106	
Pollen Viability-Shape, 120 minutes (% of pollen with collapsed walls)	Mean	99.1	98.3	
	Range	90 - 100	85 - 100	
	Confidence Interval	NA	NA	90 - 100
	Adjusted P-Value	--	NA	
	P-Value	--	NA	
Pollen Viability-Color, 0 minutes (% of pollen yellow in color)	Mean	14.4	13.2	
	Range	0 - 60	0 - 90	
	Confidence Interval	3.7 - 32.1	3.1 - 30.4	0 - 80
	Adjusted P-Value	--	0.636	
	P-Value	--	0.597	
Pollen Viability-Color, 30 minutes (% of pollen yellow in color)	Mean	68.1	66.6	
	Range	20 - 100	25 - 100	
	Confidence Interval	46.0 - 90.2	44.5 - 88.7	20 - 100
	Adjusted P-Value	--	0.636	
	P-Value	--	0.561	
Pollen Viability-Color, 60 minutes (% of pollen yellow in color)	Mean	91.4	89.7	
	Range	60 - 100	50 - 100	
	Confidence Interval	NA	NA	50 - 100
	Adjusted P-Value	--	0.534	
	P-Value	--	0.312	
Pollen Viability-Color, 120 minutes (% of pollen yellow in color)	Mean	99.7	99.4	
	Range	95 - 100	95 - 100	
	Confidence Interval	NA	NA	95 - 100
	Adjusted P-Value	--	NA	
	P-Value	--	NA	

Table 27. Across-Site Analysis of DP56113 SPTA Maintainer Agronomic Characteristics (continued)

Agronomic Characteristic	Reported Statistics	Control Maize	DP56113 SPTA Maintainer	Reference Data Range
Plant Height (cm)	Mean	201.0	197.9	126.4 - 266.2
	Range	171.6 - 252.0	156.8 - 237.4	
	Confidence Interval	183.5 - 218.6	180.4 - 215.5	
	Adjusted P-Value	--	0.276	
	P-Value	--	0.0518	
Days to Maturity	Mean	134.5	133.9	111 - 159
	Range	114 - 159	111 - 159	
	Confidence Interval	123.5 - 145.5	122.9 - 145.0	
	Adjusted P-Value	--	0.598	
	P-Value	--	0.486	
Lodging (%)	Mean	1.3	1.7	0.0 - 63.2
	Range	0.0 - 6.5	0.0 - 13.7	
	Confidence Interval	NA	NA	
	Adjusted P-Value	--	0.588	
	P-Value	--	0.441	
Final Population (count/m^2)	Mean	5.5	5.4	2.3 - 6.4
	Range	4.0 - 6.2	4.4 - 6.1	
	Confidence Interval	5.1 - 5.8	5.0 - 5.7	
	Adjusted P-Value	--	0.534	
	P-Value	--	0.367	
Ear Count (count)	Mean	1.1	1.1	1 - 3
	Range	1 - 2	1 - 2	
	Confidence Interval	NA	NA	
	Adjusted P-Value	--	0.877	
	P-Value	--	0.877	
Harvest Seed Moisture (%)	Mean	19.8	19.3	12.7 - 29.0
	Range	13.1 - 24.3	12.2 - 32.8	
	Confidence Interval	16.7 - 23.2	16.2 - 22.7	
	Adjusted P-Value	--	0.534	
	P-Value	--	0.320	
Yield (bu/A)	Mean	97.6	74.3	11 - 155
	Range	39 - 130	18 - 144	
	Confidence Interval	75.2 - 120.1	51.9 - 96.8	
	Adjusted P-Value	--	0.0241†	
	P-Value	--	0.00168*	
100-Kernel Weight (g)	Mean	30.1	31.1	21.3 - 40.4
	Range	24.7 - 36.4	25.4 - 36.0	
	Confidence Interval	28.0 - 32.2	29.0 - 33.1	
	Adjusted P-Value	--	0.0241†	
	P-Value	--	0.00301*	

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

VIII-C. Biotic and Abiotic Stressor Measurement of DP56113 SPTA Maintainer

DP56113 maize has been evaluated for response to biotic and abiotic stressors in field tests located in the United States and Puerto Rico.

Experiment A – 2017 DP56113 SPTA Maintainer Field Trial Biotic and Abiotic Stressor Measurement

Data were collected from the T4 generation of DP56113 SPTA maintainer and control maize during the 2017 growing season at eight sites in commercial maize-growing regions of the United States (two sites in Iowa, one site in Illinois, Indiana, Minnesota, Nebraska, Pennsylvania, and Washington). For each trial site, a survey of the naturally occurring insects, diseases, and abiotic stressors were recorded at four observation periods. These observations provide a means to determine if DP56113 maize responds differently from conventional maize lines to insects, diseases, abiotic stressors in the environment.

Observations from field trials demonstrated that DP56113 maize did not exhibit any unexpected responses to naturally occurring insects or diseases, and abiotic stressors as summarized in Appendix 9. These results support the conclusion that DP56113 maize is comparable to control maize lines with similar genetics or to conventional maize lines with respect to insect, disease, and abiotic stressor response.

Experiment B – DP56113 SPTA Maintainer 2014-2019 Field Observation Data

DP56113 SPTA Maintainer has been field tested in the United States and Puerto Rico over 5 years (2014-2017, 2019), as authorized by USDA-APHIS permits and notifications. For each trial, a survey of the naturally occurring insects and diseases and any unexpected differences in the response of DP56113 SPTA maintainer as compared to the control line were recorded at least every four weeks. A summary of these surveys for each trial and any differences seen between DP56113 SPTA maintainer and control lines are presented in Appendix 9. These observations provide a means to determine if DP56113 SPTA maintainer responds differently from conventional maize lines to insects or diseases in the environment.

In every case, DP56113 SPTA maintainer did not exhibit any unexpected responses to naturally occurring insects or diseases. These results, taken with the results presented above, support the conclusion that DP56113 SPTA maintainer is comparable to control maize lines with similar genetics or to non-GE conventionally bred maize lines with respect to insect or disease response.

VIII-D. Conclusions on Agronomic Performance and Field Observations of DP56113 SPTA Maintainer

DP56113 SPTA maintainer was observed in laboratory experiments and at 8 field locations in the United States and Puerto Rico to measure agronomic parameters and abiotic and biotic stressors. These experiments and field studies evaluate the characteristics of maize over a broad range of environmental conditions that represent regions where DP56113 SPTA maintainer is anticipated to be grown. The agronomic parameters measured are characteristic traits for reproduction, survival, and potential weediness.

Agronomic data demonstrated no significant differences between DP56113 SPTA maintainer and control maize with respect to early population, vegetative growth, reproductive parameters, yield, and pest responses. A statistically significant difference, before and after the FDR-adjustment, between DP56113 SPTA maintainer and the control maize was observed in the across-site analysis for yield and 100-kernel weight. For yield and 100-kernel weight, all values for DP56113 SPTA maintainer were within the reference data range.

Observations from United States and United States territory field trials over multiple years showed no unexpected differences in the response of DP56113 SPTA maintainer and control maize to naturally occurring insects and diseases. These results support the conclusion that DP56113 SPTA maintainer is comparable to control maize lines with similar genetics and/or to conventionally bred non-GE maize lines.

Based on these analyses, DP56113 SPTA maintainer is comparable to conventional maize and is unlikely to pose a greater plant pest risk or increased weed potential compared to conventionally bred non-GE maize.

IX. Potential Environmental Impact of the Introduction of DP56113 SPTA Maintainer

The potential environmental impact of a GE plant needs to be considered in the context of the characteristics of the recipient crop, the introduced trait, and the environment in which it is anticipated to be introduced (OECD, 1993). Knowledge in each of these areas provide background on which a risk or safety assessment can be made about the environmental release of the GE plant (OECD, 1993). Weediness, gene transfer or flow, and trait effects are particular issues that may be relevant to evaluating the new GE line and its safety (OECD, 1993).

To evaluate the potential environmental impact of the introduction of DP56113 SPTA maintainer, the potential for DP56113 SPTA maintainer to become weedy or invasive, the potential for gene

flow to sexually compatible wild relatives, and the potential impacts of the introduced ZM-AA1 and DsRed2 proteins were considered. As described further below, in each case, it is not anticipated that DP56113 SPTA maintainer will adversely impact the environment with respect to these considerations.

IX-A. Potential for DP56113 SPTA Maintainer to Have Altered Disease and Unintended Pest Susceptibilities or to Become Weedy or Invasive

In evaluating the potential for DP56113 SPTA maintainer to become more weedy or invasive than conventional maize, general maize biology was considered. Maize is a cultivated annual plant that generally cannot survive temperatures below freezing and is typically grown in temperate regions (OECD, 2003). Maize is not classified as a weed, is not on the United States federal or state noxious weed lists, and possesses few characteristics of notably successful weeds (Baker, 1974; Keeler, 1989; USDA-NRCS, 2011). Therefore, the natural characteristics of maize do not indicate a high potential for weediness or invasiveness.

A comparative assessment of DP56113 SPTA maintainer was conducted to determine if the DNA insertion altered the agronomic characteristics of maize. Agronomic comparison data were collected on DP56113 SPTA maintainer in multiple location field trials as described in Section VIII. This analysis showed that DP56113 SPTA maintainer was comparable to conventional maize and was comparable in agronomics. In the agronomic analyses, plant characteristics were measured, including certain endpoints that may be indicative of weediness: germination and emergence (germination rate, early stand count); reproductive characteristics (days to flowering, days to maturity, pollen viability, dropped ears, yield, and 100-kernel weight); vegetative characteristics (final population, lodging, plant height); and pest response (abiotic and biotic stressors). Characteristics related to seed germination, seed production, reproductive time and vegetative competitiveness have been identified with successful weeds (Baker, 1974). Changes to these parameters relative to the conventional variety could indicate a change in the potential weediness of a crop. DP56113 SPTA maintainer was comparable to conventional maize in each of these characteristics, indicating that DP56113 SPTA maintainer is unlikely to become more weedy or invasive than conventional maize.

In addition, DP56113 SPTA maintainer has been field tested over multiple years in multiple locations that provide a range of environmental conditions and include regions representative of maize cultivation in the United States. These fields were frequently monitored by expert growers for the incidence of diseases and insects and the effect of these on DP56113 SPTA maintainer and control plants. In all cases, no unexpected differences were observed between DP56113 SPTA maintainer and the control comparators.

In summary, DP56113 SPTA maintainer is unlikely to become more weedy or invasive than conventional maize when cultivated. Agronomic comparisons indicate no unexpected effects of the presence of the introduced proteins that alter the weediness potential of maize. No unexpected differences were detected between DP56113 SPTA maintainer and control maize in response to insects and diseases. Furthermore, the expression of the introduced ZM-AA1 and DsRed2 proteins is unlikely to increase the potential of DP56113 SPTA maintainer to become weedy.

IX-B. Potential for Gene Flow Between DP56113 SPTA Maintainer and Sexually Compatible Wild Relatives

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

DP56113 SPTA maintainer is expected to be cultivated similarly to other conventional maize varieties; therefore, it is appropriate to examine maize pollination biology, regions of maize cultivation in the United States and the geographic distribution of sexually compatible wild relatives to determine the potential for gene flow. The regions of maize cultivation in the United States and the genetic compatibility and geographic distribution of sexually compatible wild relatives of maize, within the genera *Zea* and *Tripsacum*, are discussed further below. Based on this information, there is low potential for gene flow between DP56113 maintainer and its wild relatives of the genera *Zea* and *Tripsacum* in the United States.

The potential for the insertion in DP56113 SPTA maintainer to become widespread in wild relative populations is also unlikely. The insertion does not make DP56113 SPTA maintainer more weedy than conventional cultivated maize; furthermore, none of the sexually compatible wild relatives are listed as noxious weeds.

Pollination Biology of Maize and Impact on Gene Flow

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

No significant differences were observed between the non-GE fertile pollen from DP56113 SPTA maintainer maize and conventional maize in pollen viability via measurements of shape and color over time. Pollen viability of DP56113 SPTA maintainer is comparable and no difference in pollination biology is expected when compared to conventional maize.

Regions of Maize Cultivation in the United States

Field maize is a major crop worldwide, but represents the largest crop grown in the United States. It is grown in most states, with production concentrated in the Heartland region (including Illinois, Iowa, Indiana, eastern portions of South Dakota and Nebraska, western Kentucky and Ohio, and the northern two-thirds of Missouri). Iowa and Illinois are the top maize-producing states and typically account for slightly more than one-third of the United States crop (USDA-ERS, 2009). Figure 22 indicates acres planted in the United States by county (USDA-NASS, 2011).

Additional maize varieties include popcorn and sweet corn, both of which are minor crops compared to field maize (OECD, 2002). While the range of cultivation of popcorn and sweet maize include the entire United States, in total all acreage represents less than 1% of the acreage of field maize in 2007 (USDA-NASS, 2009).

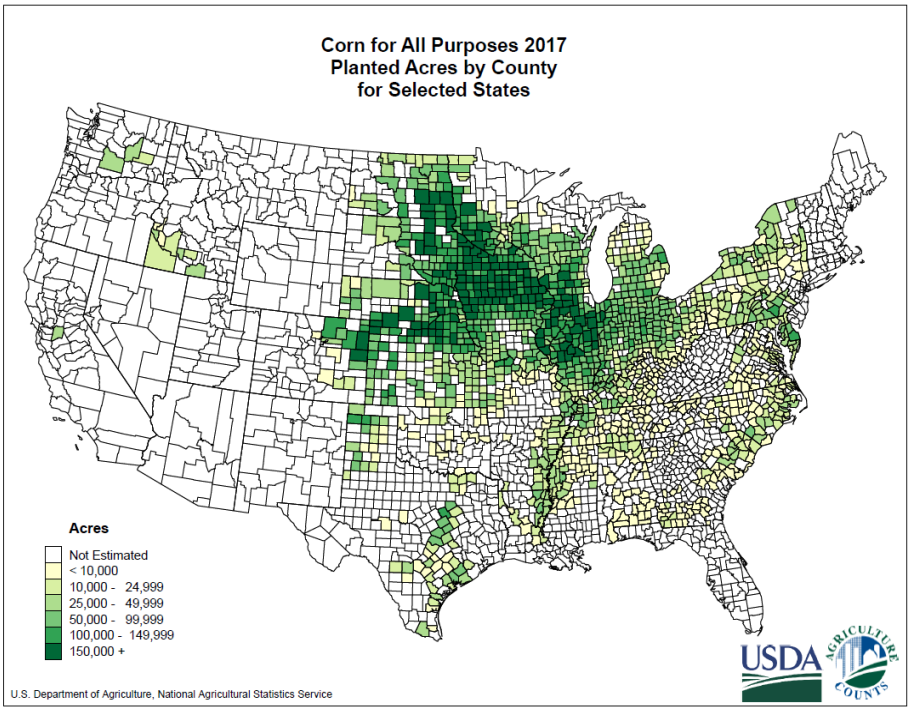


Figure 22. 2017 Corn Planted Acres - USDA-NASS (2017)

Taxonomic Classification of Maize and Related Wild Relatives

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

Potential for Gene Flow with the Genus *Zea*

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

Potential for Gene Flow with the Genus *Tripsacum*

Plants of the genus *Tripsacum* are mostly found in Mexico, Central, and South America (OECD, 2003). Three of these species (*T. dactyloides, T. floridanum*, and *T. lanceolatum*) exist as native species populations in the continental United States; and two species (*T. fasciculatum* and *T. latifolium*) were introduced in Puerto Rico (USDA, 2011). *T. dactyloides* occurs throughout the eastern half of the United States *T. lanceolatum* occurs in Arizona and New Mexico (USDA, 2011) and *T. floridanum* is native to southern Florida (USDA, 2011). Although it is extremely difficult, *Tripsacum* species (*T. dactyloides*, *T. floridanum*, and *T. lanceolatum*) can be crossed with maize;

however, hybrids have a high degree of sterility and are genetically unstable (OECD, 2003). Successful crosses of maize with *Tripsacum* species have been made experimentally, however such crosses are not known to occur in the wild (OECD, 2003). Therefore, gene flow between cultivated maize and relatives of the genus *Tripsacum* is highly unlikely.

Conclusions on the Potential for Gene Flow between DP56113 SPTA Maintainer and Wild Relatives

The potential for gene flow between maize and relatives of the genera *Zea* and *Tripsacum* is very low. While wild native or introduced populations of these genera occur where maize is cultivated, limited geographic range and low fitness or sterility of hybrids prevent successful gene flow. Furthermore, none of these wild relatives are considered to be noxious weeds and DP56113 SPTA maintainer does not exhibit greater potential for weediness as determined from agronomic comparisons to conventional maize. Therefore, any incidental gene flow between DP56113 SPTA maintainer maize and its wild relatives are unlikely to transform maize wild relatives into more weedy species, nor is it likely that the introduced trait would be introgressed widely in wild relative populations.

X. Adverse Consequences of Introduction

The data and information presented in this petition demonstrate that DP56113 SPTA maintainer is unlikely to pose a plant pest risk as compared to conventional maize. The analysis of molecular data confirmed the insertion of a single copy of the intended insertion, containing the Ms44 amiRNA cassette, and the ZM-AA1 and DsRed2 protein expression cassettes. The intended insert is stably integrated at a single locus and follows Mendelian inheritance principles over multiple breeding generations.

Evaluation of seed germination and viability characteristics showed comparability to conventional maize controls. Agronomic characteristics inclusive of plant growth and development, reproductive, and vegetative parameters were comparable to non-GE conventional maize.

Measurement of response to biotic and abiotic stressors, insects, or disease also show comparability when compared to conventional maize. This dataset supports the conclusion that DP56113 SPTA maintainer is unlikely to have an adverse impact on non-target or beneficial organisms.

Introduction of DP56113 SPTA maintainer is not anticipated to impact cultivation practices, including the management of insects, weeds, or diseases in current maize production.

The data and information contained herein supports the conclusion that DP56113 SPTA maintainer does not present a greater plant pest risk than conventional maize varieties and is not otherwise deleterious to the environment. Therefore, Pioneer requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that DP56113 SPTA maintainer, DP56113 SPTA maintainer progeny, and any crosses of DP56113 SPTA maintainer with other nonregulated maize no longer be considered regulated articles under 7 CFR §340, and that APHIS consider this petition as an extension to petition 08-338-01p. Maize DP-32138-1 (32138 SPT maintainer) was presented in 08-338-01p and received a determination of non-regulated status on May 24, 2011. DP56113 SPTA maintainer exhibits the same phenotype as 32138 SPT maintainer: male sterility conferred by the silencing of the Ms44 protein to promote pollen ablation and alpha-amylase (ZM-AA1) protein expression to deplete pollen starch supply and expression of the DsRed2 protein as a visible sorting marker.

Appendices

Appendix 1. DP56113 SPTA Maintainer USDA Release Permits, Notifications, and Planted Acreage

Year	Permit Name	Permit Valid Date	State/Territory	Number of Plantings	Acreage
2014	14-092-103n	5/1/2014	HI	2	0.002
2015	15-012-104n	3/1/2015	HI	11	0.064
2016	16-039-106n	3/1/2016	CA	3	0.060
			IL	2	0.067
			IA	4	0.083
2017	16-293-101rm-a1	11/10/2016	PR	1	0.013
	17-019-105rm	3/9/2017	IL	1	0.023
			IN	1	0.023
			IA	1	0.023
			MN	1	0.023
			NE	1	0.023
			PA	1	0.023
			WA	1	0.023
	17-038-103rm-a1	3/9/2017	IA	1	0.046
	17-263-106rm	11/6/2017	HI	1	0.161
2019	19-091-106rm	4/26/2019	NE	1	0.680
	19-105-102r *	5/8/2019	NE	1	0.680

Appendix 2. Methods for Southern by Sequencing (SbS) Analysis of DP56113 SPTA Maintainer

The test substance in this study was defined as seed from the T1 generation of DP56113 SPTA maintainer. All seed was obtained from Pioneer (Johnston, IA) and pedigree information is on file with staff breeders.

The control substance was defined as untransformed PHH5G maize seed that did not contain the DP56113 SPTA maintainer event (referred to as control maize). The unmodified line has a genetic background representative of the test substance background; however, it does not contain the DP56113 SPTA maintainer insertion. All seed were obtained from Pioneer and pedigree information is on file with staff breeders.

Plasmid DNA of PHP70533 (Figure 3) that was used for *Agrobacterium*-mediated transformation to produce DP56113 SPTA maintainer was defined as the reference substance. Plasmid dilutions used in SbS analysis were prepared from the reference substance.

Plant Growth and Sample Collection

Test and control substance (DP56113 SPTA maintainer and control maize) seeds were planted and grown and leaf tissue was collected. The leaf samples used for DNA extraction and SbS analysis were maintained frozen (≤ -50 °C) until processing.

Polymerase Chain Reaction Analysis of Plants

After germination and prior to tissue sampling for DNA extraction, plants were analyzed by PCR with an event-specific assay for the DP56113 SPTA maintainer insertion and assays for *zm-aa1*, *DsRed2*, and the *zm-Ms44* amiRNA cassette. Control maize plants were negative for all assays. Of the ten DP56113 SPTA maintainer plants from the T1 generation, one plant was positive for all assays and thus contained the inserted PHP70533 T-DNA, while the remainder were negative for all assays, indicating they did not contain the insertion.

DNA Extraction and Quantitation

The single positive T1 plant of DP56113 SPTA maintainer was used for SbS analysis along with a control maize plant. Genomic DNA was extracted from leaf tissue of DP56113 SPTA maintainer and control maize plants. The tissue was lyophilized and pulverized in tubes using a Geno/Grinder™ (SPEX CertiPrep, Inc.) instrument. Genomic DNA was isolated using Cetyltrimethylammonium bromide extraction buffer followed by purification with a Genomic-tip 100/G column (QIAGEN). Following extraction, the DNA was quantified on a spectrofluorometer

using PicoGreen® reagent (Molecular Probes, Inc.) and visualized on an agarose gel to confirm values from PicoGreen® analysis and to determine the DNA quality (Figure 6, Step 1)

Southern-by-Sequencing

SbS was performed by DuPont Pioneer Genomics Technologies. SbS analysis utilizes probe-based sequence capture, NGS techniques, and bioinformatics procedures to capture, sequence, and identify inserted DNA within the maize genome (Zastrow-Hayes et al., 2015). By compiling a large number of unique sequencing reads and mapping them against the linearized transformation plasmid and control maize genome, unique junctions due to inserted DNA are identified in the bioinformatics analysis and used to determine the number of insertions within the plant genome, verify insertion intactness, and confirm the absence of plasmid backbone sequences. The T1 generation of DP56113 SPTA maintainer was analyzed by SbS to determine the insertion copy number and intactness. SbS was also performed on control maize DNA and a positive control sample (control maize DNA spiked with PHP70533 plasmid DNA at a level corresponding to one copy of PHP70533 per copy of the maize genome) to confirm that the assay could reliably detect plasmid fragments within the genomic DNA.

The following processes were performed by Pioneer Genomics Technologies using standard methods, and were based on the procedures described in Zastrow-Hayes et al. (2015).

Capture Probe Design and Synthesis

Biotinylated capture probes used to select PHP70533 plasmid sequences were designed and synthesized by Roche NimbleGen, Inc. The probe set was designed to target all sequences within the PHP70533 transformation plasmid (Figure 6, Step 2).

Sequencing Library Construction

NGS libraries were constructed for DNA samples from individual maize plants, including DP56113 SPTA maintainer, a control maize plant, and the positive control sample. Genomic DNA purified as described above was sheared to an average fragment size of 400 bp using an ultrasonicator. Sheared DNA was end-repaired, A-tailed, and ligated to NEXTflex-HT™ Barcode adaptors (Bioo Scientific Corp.) following the kit protocol so that samples would be indexed to enable identification after sequencing. The DNA fragment libraries were amplified by PCR for eight cycles prior to the capture process. Amplified libraries were analyzed using a fragment analyzer and diluted to 5 ng/µl with nuclease-free water (Figure 6, Step 3).

Probe Hybridization and Sequence Enrichment

A double capture procedure was used to capture and enrich DNA fragments that contained sequences homologous to the capture probes. The genomic DNA libraries described above were

mixed with hybridization buffer and blocking oligonucleotides corresponding to the adapter sequences and denatured. Following denaturation, the biotinylated probes were added to the genomic DNA library and incubated at 47 °C for 16 hours. Streptavidin beads were added to the hybridization mix to bind DNA fragments that were associated with the probes. Bound fragments were washed and eluted, PCR-amplified for five cycles, and purified using spin columns. The enriched DNA libraries underwent a second capture reaction using the same conditions to further enrich the sequences targeted by the probes. This was followed by PCR amplification for 16 cycles and purification as described above. The final double-enriched libraries were quantified and diluted to 2 nM for sequencing (Figure 6, Step 4).

Next Generation Sequencing on Illumina Platform

Following sequence capture, the libraries were submitted for NGS to a depth of 100x for the captured sequences. The sequence reads were trimmed for quality below Q20 (Ewing and Green, 1998; Ewing et al., 1998) and assigned to the corresponding individual plant based on the indexing adapters. A complete sequence set from each plant is referred to as "AllReads" for bioinformatics analysis of that plant (Figure 6, Step 5).

Quality Assurance of Sequencing Reads

The adapter sequences were trimmed from the NGS sequence reads with custom scripts. Further analysis to eliminate sequencing errors used JELLYFISH, version 1.1.4 (Marçais and Kingsford, 2011), to exclude any 31 bp sequence that occurred less than twice within "AllReads" as described in Zastrow-Hayes et al. (2015). This set of sequences was used for further bioinformatics analysis and is referred to as "CleanReads". Identical sequence reads were combined into non-redundant read groups while retaining abundance information for each group. The read group sequences from the most abundant 60% of the non-redundant groups (referred to as "Non-redundantReads") were used for further analysis, as described in Zastrow-Hayes et al. (2015) (Figure 6, Step 6).

Filtering Reads

Each set of "Non-redundantReads" was aligned to the maize reference genome using Bowtie, version 1.0.0 (Langmead et al., 2009) with up to two mismatches allowed. The "Non-redundantReads" not matching the maize reference genome were then compared to the PHP70533 T-DNA sequence using Bowtie with zero mismatches allowed. Any "Non-redundantReads" that were not wholly derived from either sequence were aligned to the PHP70533 plasmid backbone with Bowtie 2, version 2.1.0, allowing zero mismatches. The ubiquitous presence of environmental bacteria, such as *Serratia marcescens,* provides an opportunity for their plasmid DNA to be sequenced along with plant genomic DNA. This resulted in low level detection of PHP70533 plasmid backbone sequences in the genomic DNA samples

due to similarity with the PHP70533 backbone region. "Non-redundantReads" that aligned to the PHP70533 backbone sequence, but at a coverage depth below 35x across 50 bp, were deemed to be due to environmental bacteria (Figure 6, Step 7). Due to the detection of these bacterial sequences, coverage levels of 35x or below were considered to be the background level of sequencing

Junction Detection

Following removal of "Non-redundantReads" with alignments wholly to the maize reference genome or T-DNA sequence identified during the quality assurance phase, the remaining "Non-redundantReads" were aligned to the full PHP70533 plasmid sequence using BWA, version 0.5.9-r16, with the soft-trimming feature enabled (Li and Durbin, 2010). Chimeric reads contain sequence that is non-contiguous with the PHP70533 sequence from the alignment, such as genome-plasmid junctions or rearrangements of the plasmid. These chimeric reads are referred to as junction reads or junctions. The individual reads defining a junction were condensed to a unique identifier to represent the junction. This identifier (referred to as a 30_20 mer) includes 20 bp of sequence from PHP70533 and 30 bp of sequence adjacent to the 20 bp from the plasmid. The adjacent 30 bp did not align to PHP70533 contiguously to the known 20 bp. When the 20 bp from PHP70533 and the adjacent 30 bp are combined into a 30_20 mer, they indicate the junction shown by the chimeric read. Junction reads were condensed into a unique junction if their 30_20 mers were identical, or if the 30_20 mer junctions were within 2 bp. The total number of sequence reads (referred to as "TotalSupportingReads") for each unique junction was retained for filtering. Junctions with fewer than five unique supporting reads, or if the "TotalSupportingReads" value was equal to or below 10% of the median sequencing depth for positions aligned to the plasmid, were filtered and removed from further analysis (Figure 6, Step 8).

Junction Identification

Variations between the maize reference genome used in the SbS analysis and the control maize genome may result in identification of junctions that are due to these differences in the endogenous maize sequences. In order to detect these endogenous junctions, control maize genomic DNA libraries were captured and sequenced in the same manner. These libraries were sequenced to an average depth approximately five times the depth for the DP56113 SPTA maintainer plant sample. This increased the probability that the endogenous junctions captured by the PHP70533 probes would be detected in the control maize samples, so that they could be identified and removed from the DP56113 SPTA maintainer sample. The 30_20 mers of the endogenous junctions detected in this analysis were used to filter the same endogenous junctions in the DP56113 SPTA maintainer samples (Figure 4, Step 8), so that the only junctions

remaining in the DP56113 SPTA maintainer sample are due to actual PHP70533 insertions (Figure 6, Step 9).

Appendix 3. Materials and Methods for Southern Blot Analysis of DP56113 SPTA Maintainer

Test Substance

The test substance in this study was defined as seed from four generations of DP56113 SPTA maintainer. All seed was obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA) and pedigree information is on file with staff breeders.

Control Substance

The control substance was defined as untransformed PHH5G maize seed that did not contain the DP56113 SPTA maintainer event (referred to as control maize). The unmodified line has a genetic background representative of the test substance background; however, it does not contain the DP56113 SPTA maintainer insertion. All seed were obtained from Pioneer Hi-Bred International, Inc. and pedigree information is on file with staff breeders.

Reference Substance

Plasmid DNA of PHP70533 (Figure 3) that was used for *Agrobacterium*-mediated transformation to produce DP56113 SPTA maintainer was defined as the reference substance. Plasmid dilutions used in Southern blot analysis were prepared from the reference substance.

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were obtained from commercial vendors and used to determine approximate molecular weights of DNA fragments. For Southern blot analysis, digoxigenin (DIG)-labeled DNA Molecular Weight Markers II and VII (Roche) were used as size standards for hybridizing fragments.

Plant Growth and Sample Collection

Test and control substance (DP56113 SPTA maintainer and control maize) seeds were planted and grown and leaf tissue was collected. The leaf samples used for DNA extraction and Southern blot analysis were maintained frozen (≤ -50 °C) until processing.

Polymerase Chain Reaction Analysis of Plants

After germination and prior to tissue sampling for DNA extraction, plants were analyzed by polymerase chain reaction (PCR) with an event-specific assay for the DP56113 SPTA maintainer insertion and assays for *zm-aa1*, *DsRed2*, and the *zm-Ms44* amiRNA cassette. Control maize plants were negative for all assays. DP56113 SPTA maintainer plants from each of the four

generations that were positive for all assays, and thus contained the inserted DNA, were selected for DNA extraction.

DNA Extraction and Quantitation

Genomic DNA was extracted from leaf tissue of DP56113 SPTA maintainer plants from four generations and control maize plants. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep, LLC.) and the genomic DNA was isolated using a standard Urea Extraction Buffer procedure. Following extraction, the DNA was quantified on a spectrofluorometer using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes, Inc.).

Digestion of DNA and Electrophoretic Separation

All restriction enzymes were obtained from New England BioLabs. Genomic DNA samples extracted from test and control plants were digested with *Bmt* I or *Sca* I for analysis of the borders of the DP56113 SPTA maintainer insertion. Plasmid PHP70533 was added to control plant DNA at a level equivalent to one copy of plasmid per genomic copy and digested with either *Bmt* I or *Sca* I to confirm hybridization of the probes used for the Southern blot analysis.

Following digestion with the restriction enzymes, the fragments produced were electrophoretically separated by size through an agarose gel, and a molecular weight standard, ΦX174 RF DNA/*Hae* III Fragments (Invitrogen), was used to determine sufficient migration and separation of the fragments on the gel.

Southern Transfer

Agarose gels containing the separated DNA fragments were depurinated, denatured, and neutralized *in situ*, and transferred to a nylon membrane in 20x SSC buffer using the method as described for the TURBOBLOTTER™ Rapid Downward Transfer System (GE Healthcare Bio-Sciences). Following transfer to the membrane, the DNA was bound to the membrane by UV crosslinking (Stratalinker, Stratagene).

Probe Labeling and Southern Blot Hybridization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized with a labeled probe. The probes used for Southern hybridization are provided in Table 1. Fragments corresponding to the *zm-aa1* gene, *DsRed2* gene, and the *Ms44* amiRNA cassette were amplified by PCR from plasmid PHP70533. All probes were prepared from the corresponding gel-purified fragments by PCR that incorporated a DIG-labeled nucleotide, (DIG-11)-dUTP, according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

Labeled probes were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the procedures described for DIG Easy Hyb solution (Roche). The *zm-aa1* probe was hybridized to the *Bmt* I digest to detect the 5' border of the DP56113 SPTA maintainer insertion, while the *DsRed2* and *Ms44* probes were hybridized individually to the *Sca* I digest to detect the 3' border of the insertion. DIG labeled DNA Molecular Weight Markers II and VII (Roche), visible after DIG detection as described below, were used to determine hybridizing fragment size on the Southern blots.

Detection of Hybridized Probes

DIG-labeled probes, hybridized to DNA bound to the nylon membrane after stringent washes, and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Images were digitally captured by detection with the ImageQuant LAS 4000 (GE Healthcare Bio-Sciences). Detected bands were documented for each digest and each probe.

Stripping of Probes and Subsequent Hybridizations

Following hybridization and detection, the membranes were stripped of DIG-labeled probe to prepare the blot for subsequent re-hybridization if required. Membranes were rinsed briefly in distilled de-ionized water and stripped in a solution of 0.2 M NaOH and 0.1% sodium dodecyl sulfate (SDS) at 37°C with constant shaking. The membranes were then rinsed in 2x saline sodium citrate (SSC) and either used directly for subsequent hybridizations or stored for later use. The alkali-based stripping procedure effectively removes probes labeled with the alkali-labile DIG used in hybridizations.

Appendix 4. Methods and Materials for Segregation Analysis of Five Generations of DP56113 SPTA Maintainer Maize

Experimental Design

Segregation analysis was conducted for five generations of DP56113 SPTA maintainer (T2, T3, T4, T5, and F1). Genotypic analyses were used to evaluate each individual plant for the presence or absence of event DP56113, the *zm-Ms44* amiRNA, and the *zm-aa1* and *DsRed2* genes by qualitative polymerase chain reaction. Statistical analysis (chi-square test at 0.05 significance level) was conducted to compare the observed segregation ratio to the expected segregation ratio of 1:1 for each generation.

Leaf tissue samples were collected from five generations of DP56113 SPTA maintainer (T1, T2, T3, T4, and F1) and anon-GE near-isoline maize (referred to as control maize) at various growth stages and made available for use in future analysis.

Bias in this study was controlled through uniform maintenance of all entries during the entire study period.

Planting and Thinning

For the six generations of DP56113 SPTA maintainer (T1, T2, T3, T4, T5, and F1), 120-135 seeds were planted per generation in separate 4-inch pots (one seed per pot, organized in flats containing 15 pots). For the control maize, 10 seeds were planted in a 2-gallon pot. All seeds were grown in a controlled environment under conditions for producing maize plants. Each generation of DP56113 SPTA maintainer maize was then thinned to at least 100 plants for genotypic evaluation by removing any unhealthy plants.

Segregation Analysis

PCR Sample Collection

For five generations of DP56113 SPTA maintainer (T2, T3, T4, T5, and F1), one leaf sample per plant was collected at the V3 growth stage to be used in PCR analysis. Each sample consisted of three leaf punches collected into one bullet tube and placed on dry ice until transferred to a freezer prior to analysis. Individual plants and corresponding leaf samples were uniquely labeled to allow a given sample to be tracked back to the originating plant.

Genotype Analysis

Leaf samples were analyzed using a qualitative PCR assay to confirm the presence or absence of event DP56113, the *zm-Ms44* amiRNA, and *zm-aa1* and *DsRed2* genes.

Leaf Punch Sample Collection

Four leaf punch samples were collected at the V5 growth stages from each plant. The plants were uniquely labeled to allow a given sample to be traced back to the originating plant. Samples were placed in coolers on dry ice immediately after sampling and kept frozen until transferred to a freezer set at ≤ -50 ºC.

Statistical Analysis

A chi-square test was performed at the 0.05 significance level on the segregation results of T2, T3, T4, T5 and F1 generations of DP56113 SPTA maintainer. The chi-square test was performed separately for each generation to compare the observed segregation ratio to the expected segregation ratio of 1:1 for each generation. Statistical analyses were conducted using SAS software, Version 9.4.

Appendix 5. Methods and Materials for Determination of ZM-AA1 and DsRed2 Protein Concentrations in DP56113 SPTA Maintainer

Experimental Design

The field portion of this study was conducted during the 2017 growing season at eight sites in commercial maize-growing regions of the United States (two sites in Iowa and one site each in Illinois, Indiana, Minnesota, Nebraska, Pennsylvania, and Washington). A randomized complete block design with four blocks (each containing DP56113 SPTA maintainer) was utilized at each site.

The following samples were collected: leaf (V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), forage (R4 growth stage), root (V9, R1, R4, and R6 growth stages), whole plant (V9, R1, and R6 growth stages), and seed (R6 growth stage). Growth stage descriptions are provided in Table 1. Samples collected from DP56113 SPTA maintainer were analyzed for ZM-AA1 protein concentrations using a quantitative enzyme-linked immunosorbent assay (ELISA) and DsRed2 protein concentrations using a fluorometric assay method.

Bias in this portion of the study was controlled through the use of replicate testing, appropriate assay controls, and pre-determined data acceptability criteria.

Sample Collection

Leaf (V9, R1, R4, and R6 growth stages), root (V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), whole plant (V9, R1, and R6 growth stages), forage (R4 growth stage), and seed (R6 growth stage) samples were from all four blocks collected at each site from DP56113 SPTA maintainer for expressed trait protein analysis. One sample per plot was collected from a self-pollinated row for each tissue at the applicable growth stages. All samples from a given growth stage were collected from the same plants. All samples were collected from impartially selected, healthy, representative plants to minimize potential bias. Each sample was uniquely labeled with a sample identification number and barcode for sample tracking, and is traceable by site, entry, block, tissue, and growth stage.

Leaf

Each leaf sample was obtained by pruning the youngest, healthy leaf that was at least 8 in. (20 cm) in length from the plant. The tissue was cut into sections of 1 in. (2.5 cm) or smaller and collected into a pre-labeled vial.

Root

Each root sample was obtained by cutting a circle 10-15 in. (25-38 cm) in diameter around the base of the plant to a depth of 7-9 in. (18-23 cm). The roots were thoroughly cleaned with water and removed from the plant. No above ground brace roots were included in the sample. The root tissue was cut into sections of 1 in. (2.5 cm) or smaller in length and collected to fill no more than 50% of a pre-labeled vial.

Pollen

Each pollen sample was obtained by bagging and shaking a selected tassel to dislodge the pollen. The tassel selected for sampling had one-half to three-quarters of the tassel's main spike shedding pollen. For some plots, may have been pooled from multiple plants within the same plot in order to collect the appropriate amount. The pollen was screened for anthers and foreign material, and then collected to fill approximately 25-50% of the conical area of a pre-labeled vial.

Whole Plant

Each whole plant sample was obtained by cutting the plants approximately 4-6 in. (10-15 cm) above the soil surface line. The plant was chopped into sections of 3 in. (7.6 cm) or less in length and collected into a pre-labeled, plastic-lined, cloth bag. The plants selected for sampling at the R1 growth stage contained tassels and ears that were covered prior to silking (any secondary or tertiary ears with exposed silks were removed and excluded from the sample). The plants selected for sampling at the R6 growth stage contained tassels and self-pollinated ears. Any secondary or tertiary ears with exposed silks were removed from the plants selected for sampling at the R6 growth stage. The R6 whole plant samples included the husk and cob from the sampled plants; however, the seed was removed and used for the respective seed sample.

Forage

Each forage sample was obtained by cutting the aerial portions of the plants approximately 4-6 in. (10-15 cm) above the soil surface line. The plant was chopped into sections of 3 in. (7.6 cm) or less in length and collected into a pre-labeled, plastic-lined, cloth bag. The plants selected for forage sampling contained self-pollinated ears.

Seed

Each seed sample was obtained by husking and shelling the seed from one selected ear. The plants selected for seed sampling contained self-pollinated ears. For each sample, a representative sub-sample of 15 kernels was collected into an individual pre-labeled vial.

Each sample was placed on dry ice within 10 minutes of collection in the field and transferred to frozen storage (< -10 °C freezer unit) until shipment. Expressed trait protein samples were then shipped frozen to Pioneer Hi-Bred International, Inc. for processing and analysis. Upon arrival, samples were stored frozen (< -10 °C freezer unit). Prior to processing, seed samples were color-sorted using methods described in Methods section C. Whole plant and forage samples were coarsely homogenized prior to lyophilization. All samples were lyophilized under vacuum until dry. Following lyophilization, leaf, root, whole plant, forage, and seed samples were finely homogenized and stored frozen until analysis.

Color Sorting of R6 Seed Samples

Frozen kernels from each individual seed R6 sample, from all field sites, were evaluated under a light source of appropriate wave length to confirm fluorescence. All red kernels were collected and retained for tissue processing and analysis.

Protein Concentration Determination

The concentrations of ZM-AA1 protein was determined using a quantitative ELISA that has been internally validated to demonstrate method suitability. The concentration of DsRed2 protein was determined using a quantitative fluorometric assay that has been internally validated to demonstrate method suitability.

ZM-AA1 Protein Extraction

Processed tissue sub-samples were weighed at the following target weights: 5 mg for pollen; 10 mg for leaf; 20 mg for root, and seed; and 30 mg for whole plant and forage. Samples were extracted with 0.60 ml of chilled O8 buffer, which was comprised of 50 mM HEPES, 150 mM sodium chloride, 0.15% polysorbate 20, 0.5 mM calcium chloride, 0.5% polyethylene glycol, and 5 mM sodium metabisulfite. Extracted samples were centrifuged, and then supernatants were removed and prepared for analysis.

DsRed2 Protein Extraction

Processed tissue sub-samples were weighed at the following target weights: 5 mg for pollen; 10 mg for seed and leaf; 15 mg for root; and 20 mg for forage and whole plant. Samples were extracted with 0.80 ml of chilled 25% StabilZyme Select in phosphate-buffered saline containing polysorbate 20 (PBST). Extracted samples were centrifuged, and then supernatants were removed and prepared for analysis.

ZM-AA1 Protein ELISA Methodology

Prior to analysis, samples were diluted as applicable in O8 buffer. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with a ZM-AA1-specific antibody. Following incubation, unbound substances were washed from the plate and the bound ZM-AA1 protein was incubated with a different ZM-AA1-specific antibody conjugated to the enzyme horseradish peroxidase (HRP). Unbound substances were washed from the plate. Detection of the bound ZM-AA1-antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader.

DsRed2 Protein Fluorometric Assay Method

Prior to analysis, samples were diluted as applicable with 25% StabilZyme Select in PBST. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were analyzed on the same test plate. The test plate was placed into a fluorometer where the DsRed2 protein was excited with light and the resulting fluorescence intensity was measured in relative fluorescence units (RFU). Fluorometer wavelength settings were at 563 nm excitation and 600 nm emission with a 590 nm cutoff filter. The intensity of fluorescence was directly related to the amount of DsRed2 protein present in the sample extract.

Calculations for Determining ZM-AA1 Protein Concentrations

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the OD values obtained for each set of sample wells to a protein concentration value.

A standard curve was included on each ELISA plate. The equation for the standard curve was derived by the software, which used a quadratic fit to relate the OD values obtained for each set of standard wells to the respective standard concentration (ng/ml).

The quadratic regression equation was applied as follows: $y = Cx^2 + Bx + A$

where x = known standard concentration and y = respective absorbance value (OD)

Interpolation of the sample concentration (ng/ml) was performed by solving for x in the above equation using the values for A, B, and C that were determined for the standard curve.

Sample Concentration (ng/ml) = $\frac{-B + \sqrt{B^2 - 4C(A - \text{sampleOD})}}{2C}$

For example, given curve parameters of A = 0.0476, B = 0.4556, C= -0.01910, and a sample OD = 1.438

Sample Concentration = $\frac{-0.4556+\sqrt{0.4556^2-4(-0.01910)(0.0476-1.438)}}{2(-0.01910)}$ = 3.6 ng/ml

The sample concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the interpolated concentration by N.

Adjusted Concentration = Interpolated Sample Concentration x Dilution Factor

For example, given an interpolated concentration of 3.6 ng/ml and a dilution factor of 1:20

Adjusted Concentration = 3.6 ng/ml x 20 = 72 ng/ml

Adjusted sample concentration values obtained from SoftMax Pro GxP software were converted from ng/ml to ng/mg sample weight as follows:

$$\text{Sample Concentration (ng protein/mg sample weight)} = \text{Sample Concentration (ng/ml)} \times \frac{\text{Extraction Buffer Volume (ml)}}{\text{Sample Target Weight (mg)}}$$

For example, sample concentration = 72 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 10 mg

$$\text{Sample Concentration (ng protein/mg sample weight)} = 72 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{10 \text{ mg}} = 4.3 \text{ ng/mg}$$

The reportable assay lower limit of quantification (LLOQ) in ng/ml was calculated as follows:

Reportable Assay LLOQ (ng/ml) = (lowest standard concentration - 10%) x minimum dilution

For example, lowest standard concentration = 0.50 ng/ml and minimum dilution = 10

Reportable Assay LLOQ (ng/ml) = (0.50 ng/ml - (0.50 x 0.10)) x 10 = 4.5 ng/ml

The LLOQ, in ng/mg sample weight, was calculated as follows:

$$\text{LLOQ} = \text{Reportable Assay LLOQ (ng/ml)} \times \frac{\text{Extraction Buffer Volume (ml)}}{\text{Sample Target Weight (mg)}}$$

For example, reportable assay LLOQ = 4.5 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 10 mg

$$\text{LLOQ} = 4.5 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{10 \text{ mg}} = 0.27 \text{ ng/mg sample weight}$$

Calculations for Determining DsRed2 Protein Concentrations

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the RFU values obtained for each set of sample wells to a protein concentration value.

A standard curve was included on each plate. The equation for the standard curve was derived by the software, which used a quadratic fit to relate the mean RFU values obtained for each set of standard wells to the respective standard concentration (µg/ml).

The quadratic regression equation was applied as follows: $y = Cx^2 + Bx + A$

where x = known standard concentration and y = RFU value

Interpolation of the sample concentration (µg/ml) was performed by solving for x in the above equation using the values for A, B, and C that were determined for the standard curve.

$$\text{Sample Concentration (µg/ml)} = \frac{-B + \sqrt{B^2 - 4C(A - RFU)}}{2C}$$

For example, given curve parameters of A = 6.06, B = 162, C = -2.57, and a sample RFU = 520

Sample Concentration (µg/ml) = $\frac{-162+\sqrt{162^2-4(-2.57)(6.06-520)}}{2(-2.57)}$ = 3.4 µg/ml

The sample concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the sample concentration by N.

Adjusted Concentration = Sample Concentration x Dilution Factor

For example, given a sample concentration of 3.4 µg/ml and a dilution factor of 1:2

Adjusted Concentration = 3.4 µg/ml x 2 = 6.8 µg/ml

Adjusted sample concentration values obtained from SoftMax Pro GxP software were converted from µg/ml to ng/mg sample weight as follows:

$$\text{Sample Concentration (ng protein/mg sample weight)} = \text{Sample Concentration (µg/ml)} \times \frac{\text{Extraction Buffer Volume (ml)}}{\text{Sample Target Weight (mg)}} \times \frac{1000\ \text{ng}}{1\ \mu\text{g}}$$

For example, sample concentration = 6.8 µg/ml, extraction buffer volume = 0.80 ml, and sample target weight = 10 mg

$$\text{Sample Concentration (ng protein/mg sample weight)} = 6.8\ \mu\text{g/ml} \times \frac{0.80\ \text{ml}}{10\ \text{mg}} \times \frac{1000\ \text{ng}}{1\ \mu\text{g}} = 540\ \text{ng/mg}$$

The reportable assay LLOQ in µg/ml was calculated as follows:

Reportable Assay LLOQ (µg/ml) = (lowest standard concentration - 10%) x minimum dilution

For example, lowest standard concentration = 0.25 µg/ml and minimum dilution = 2

Reportable Assay LLOQ (µg/ml) = (0.25 µg/ml - (0.25 x 0.10)) x 2 = 0.45 µg/ml

The LLOQ, in ng/mg sample weight, was calculated as follows:

$$\text{LLOQ} = \text{Reportable Assay LLOQ} \times \frac{\text{Extraction Buffer Volume (ml)}}{\text{Sample Target Weight (mg)}} \times \frac{1000\ \text{ng}}{1\ \mu\text{g}}$$

For example, DsRed2 in inbred seed: reportable assay LLOQ = 0.45 µg/ml, extraction buffer volume = 0.80 ml, and sample target weight = 10 mg

$$\text{LLOQ} = 0.45\ \mu\text{g/ml} \times \frac{0.80\ \text{ml}}{10\ \text{mg}} \times \frac{1000\ \text{ng}}{1\ \mu\text{g}} = 36\ \text{ng protein/mg sample weight}$$

Statistical Analysis

Statistical analysis of the protein concentration results consisted of the calculations of means, ranges, and standard deviations. Individual sample results below the LLOQ were assigned a value equal to half of the LLOQ for calculation purposes.

Appendix 6. Methods for Protein Characterization and Equivalency Analysis

Test Substance

The test substance consisted of event DP-Ø56113-9 contained within maize seed.

The floret tissue was collected at the VT growth stage (the stage when the last branch of tassel is completely visible) and the seed tissue was collected at the R6 growth stage (typical harvest stage) (Abendroth et al., 2011) of development from Pioneer-grown plants. The tissues were lyophilized, homogenized and stored at ≤ -10 °C.

Reference Substances (Protein Analytical Standards)

Protein stability was certified under the stated storage condition for each analytical standard.

Protein Extraction

Processed tissue sub-samples were weighed to approximately 10 mg each and ZM-AA1 and DsRed2 protein reference standards were weighed to approximately 5 mg each. ZM-AA1 and DsRed2 proteins were extracted from the floret and seed tissue sub-samples, respectively, in 600 µl of 1X LDS/DTT sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water) by homogenization with a GenoGrinder. Extracted samples were centrifuged, and then supernatants were removed. The reference standards were solubilized in 1X LDS/DTT sample buffer to a target concentration of 2 mg/ml and then diluted using 1X LDS/DTT sample buffer to 5 µg/ml. All samples and standards were heated at 90-100 °C for 5 minutes and stored frozen (≤ -10 °C freezer unit).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The LDS/DTT treated samples and reference standards stored at ≤ -10 °C were re-heated for 5 minutes at 90-100 °C and then loaded into a 4-12% Bis-Tris gel. Prestained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into the gel to provide a visual verification that migration was within the range of the predicted molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES SDS running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gel was removed from the gel cassette and used for western blot analysis.

Western Blot Analysis

Following SDS-PAGE, the resulting gel was assembled into a nitrocellulose (NC) iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gel to the NC membrane for 7 minutes with a pre-set program (P3).

Following protein transfer, the membrane was blocked in PBST containing 5% weight/volume (w/v) non-fat dry milk for 45 minutes at ambient laboratory temperature. Before and after the blocking step, the membrane was washed with PBST for at least 1 minute to reduce the background. For ZM-AA1 protein detection, the blocked membrane was incubated for 60 minutes at ambient laboratory temperature with a ZM-AA1 monoclonal antibody (2A2.G3.H2-HRP) conjugated to horseradish peroxidase diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk. For DsRed2 protein detection, the blocked membrane was incubated for 60 minutes at ambient laboratory temperature with a DsRed2 polyclonal antibody (ID # 1510004) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk. Following primary antibody incubation, the blots were washed 3 times in PBST for 5 minutes each. The DsRed2 protein blot was incubated with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:20,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient temperature. The blot was then washed with PBST three times for 5 minutes each. The ZM-AA1 and DsRed2 protein blots remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

Appendix 7. Methods and Materials used to Measure Composition of Seed and Forage of DP56113 Maize

Materials

The test system in this study was maize (*Zea mays* L.). The test substance consisted of the event DP-Ø56113-9 contained within maize seed.

The control substance consisted of non-genetically modified (non-GM) near-isoline maize seed (referred to as control maize), which did not contain event DP-Ø56113-9. Additionally, a total of 14 non-GM proprietary maize lines (collectively referred to as reference maize) were included in the study as reference substances.

The procedures for identifying the test system included clearly identifying all plots and uniquely labeling all collected samples.

Methods

Experimental Design

The field portion of this study was conducted during the 2017 growing season at eight sites in commercial maize-growing regions of the United States (two sites in Iowa, one site in Illinois, Indiana, Minnesota, Nebraska, Pennsylvania, and Washington). A randomized complete block design with four blocks was utilized at each site. Each block included DP56113 SPTA maintainer, non-genetically modified (non-GM) near-isoline control maize (referred to as control maize), and four of the following non-GM proprietary maize lines: PH12K9, PHWRW, PHB00, PHR1R, PHEJW, PH134G, PH3KP, PH1M3S, PH8JR, PH24E, PHCJP, PH1DCP, PHR62, and PHCER (referred to as reference maize). Site design and crop maintenance practices are described in study phase report PHI-2017-004/001 (Pauli et al., 2018).

Bias in this portion of the study was controlled through the use of the same sample identification numbers assigned to the originally collected samples, through the use of pre-set data acceptability criteria, and through the arrangement of samples for analyses without consideration whether the samples were derived from a test, control, or reference substance.

Sample Collection

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected (one sample per plot) from Rows 5 and/or 6 at the applicable growth stage. Growth stage descriptions are provided in Table A7-1. All samples were collected from impartially selected, healthy, representative plants to minimize potential bias. Reference maize and control maize samples

were collected prior to the collection of DP56113 SPTA maintainer samples to minimize the potential for contamination.

Each forage sample (combination of three plants) was obtained by cutting the aerial portion of the plants from the root system approximately 4-6 in. (10-15 cm) above the soil surface line. The plants were chopped into sections of 3 in. (7.6 cm) or less in length, pooled, and approximately one-half of the chopped material was collected in a pre-labeled, plastic-lined, cloth bag.

Each grain sample was obtained from 10 ears at typical harvest maturity from self-pollinated or sib-pollinated plants. The ears were husked and shelled, and the pooled grain was collected into a large, plastic, resealable bag and then placed into a pre-labeled, plastic-lined, cloth bag.

Each sample was placed into chilled storage (*e.g.*, coolers with wet ice, artificial ice, or dry ice) after collection and transferred to a freezer (≤ -10 °C). Samples were shipped or delivered frozen to Pioneer Hi-Bred (Johnston, IA, USA) for storage and remained frozen.

Nutrient Composition Analyses

Nutrient composition analyses of forage and grain samples included the determination of the following analytes:

Proximate, Fiber, and Mineral Composition in Forage

- Moisture*
- Crude Protein
- Crude Fat
- Crude Fiber
- Acid Detergent Fiber (ADF)
- Neutral Detergent Fiber (NDF)
- Ash
- Carbohydrates
- Calcium
- Phosphorus

*Note: Moisture data were used to convert corresponding analyte values for a given sample to a dry weight basis, and were not included in subsequent statistical analysis and reporting of results.

Proximate and Fiber Composition in Grain

- Moisture*
- Total Dietary Fiber
- Crude Protein
- Crude Fat
- Crude Fiber
- Acid Detergent Fiber (ADF)
- Neutral Detergent Fiber (NDF)
- Ash
- Carbohydrates

*Note: Moisture data were used to convert corresponding analyte values for a given sample to a dry weight basis, and were not included in subsequent statistical analysis and reporting of results.

Fatty Acid Composition in Grain

- Lauric Acid (C12:0)
- Myristic Acid (C14:0)
- Palmitic Acid (C16:0)
- Palmitoleic Acid (C16:1)
- Heptadecanoic Acid (C17:0)
- Heptadecenoic Acid (C17:1)
- Stearic Acid (C18:0)
- Oleic Acid (C18:1)
- Linoleic Acid (C18:2)
- α-Linolenic Acid (C18:3)
- Arachidic Acid (C20:0)
- Eicosenoic Acid (C20:1)
- Eicosadienoic Acid (C20:2)
- Behenic Acid (C22:0)
- Erucic Acid (C22:1)
- Lignoceric Acid (C24:0)

Amino Acid Composition in Grain

- Alanine
- Arginine
- Aspartic Acid
- Cystine
- Glutamic Acid
- Glycine
- Histidine
- Isoleucine
- Leucine
- Lysine
- Methionine
- Phenylalanine
- Proline
- Serine
- Threonine
- Tryptophan
- Tyrosine
- Valine

Mineral Composition in Grain

1. Calcium
2. Copper
3. Iron
4. Magnesium
5. Manganese
6. Phosphorus
7. Potassium
8. Sodium
9. Zinc

Vitamin Composition in Grain

- β-Carotene
- Vitamin B1 (Thiamine)
- Vitamin B2 (Riboflavin)
- Vitamin B3 (Niacin)
- Vitamin B5 (Pantothenic Acid)
- Vitamin B6 (Pyridoxine)
- Vitamin B9 (Folic Acid)
- α-Tocopherol
- β-Tocopherol
- γ-Tocopherol
- δ-Tocopherol

Note: an additional analyte, total tocopherols, was subsequently calculated as the sum of the α-, β-, γ-, and δ-tocopherol values for each sample for use in statistical analysis and reporting of results.

Secondary Metabolite and Anti-Nutrient Composition in Grain

- *p*-Coumaric Acid
- Ferulic Acid
- Furfural
- Inositol
- Phytic Acid
- Raffinose
- Trypsin Inhibitor

Statistical Analysis

Statistical analyses were conducted to evaluate and compare the nutrient composition of forage and grain derived from DP56113 SPTA maintainer and the control maize.

Processing of Data

Values Below Lower Limit of Quantification

For statistical analysis, nutrient composition values reported as below the assay lower limit of quantification (LLOQ) were each assigned a value equal to half the LLOQ.

Conversion of Fatty Acid Assay Values

The raw data for all fatty acid analytes were provided by EPL BAS in units of percent fresh weight (%FW). Any fatty acid values below the %FW LLOQ were set to half the LLOQ value, and then all assay values were converted to units of % total fatty acids for statistical analyses.

For a given sample, the conversion to units of % total fatty acids was performed by dividing each fatty acid analyte value (%FW) by the total fresh weight of all fatty acids for that sample; for analyte values below the LLOQ, the half LLOQ value was used as the analyte value. Half LLOQ values were also included in the total fresh weight summations. After the conversion, a fixed LLOQ value was not available for a given individual fatty acid analyte on the % total fatty acids basis.

Erucic Acid (C22:1) data were excluded from the conversion and from statistical analyses because all sample values in the current study and in historical commercial reference maize lines were below the LLOQ.

Calculation of Additional Analytes

One additional analyte (total tocopherol) was calculated for statistical analyses. The total amount of tocopherol for each sample was obtained by summing the assay values of α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol in the sample.

If the assay value of an individual analyte was below the LLOQ for a given sample, half of the LLOQ value was used in computing the total. The total was considered below the LLOQ only when all the individual analytes contributing to its calculation were below the LLOQ.

Selection of Statistical Method

The following rules were implemented:

If both DP56113 SPTA maintainer and the control maize had < 50% of samples below the LLOQ, then an across-site mixed model analysis would be conducted. In addition, if both maize lines had at least two samples at a given site above the LLOQ, then an individual-site mixed model analysis would be conducted.

If either DP56113 SPTA maintainer or the control maize had ≥ 50% samples below the LLOQ, but not both entries had 100% of samples below the LLOQ across sites, then Fisher's exact test would be conducted. The Fisher's exact test assessed whether there was a significant difference (P-value < 0.05) in the proportion of samples below the LLOQ between these two maize lines across sites. Individual-site analyses would not be performed.

If both DP56113 SPTA maintainer and the control maize had 100% of samples below the LLOQ, then statistical analyses would not be performed.

Across-Site Analysis

For a given analyte, data were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ Model 1

$\ell_j \sim iid\ N(0, \sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0, \sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Ent\times Site})$, and $\varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$

where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓ_j denotes the effect of the j^{th} site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the j^{th} site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect), and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} site (random effect or residual). Notation $\sim iid\ N(0, \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a. Subscript *a* represents the corresponding source of variation.

The residual maximum likelihood estimation procedure was utilized to generate estimates of variance components and entry means across sites. The estimated means are known as empirical best linear unbiased estimators (hereafter referred to as LS-Means). The statistical comparison was conducted by testing for a difference in LS-Means between DP56113 SPTA maintainer and the control maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method (Kenward and Roger, 2009). A significant difference was identified if a P-value was < 0.05.

For each analyte, goodness-of-fit of the model was assessed in terms of meeting distributional assumptions of normally, independently distributed errors with homogeneous variance. Deviations from assumptions were addressed using an appropriate transformation or a heterogeneous error variance structure.

Individual-Site Analysis

For a given analyte, individual sites were analyzed separately using the following linear mixed model:

$y_{ik} = \mu_i + r_k + \varepsilon_{ik}$ Model 2

$r_k \sim iid\ N(0, \sigma^2_{Rep})$ and $\varepsilon_{ik} \sim iid\ N(0, \sigma^2_{Error})$

where μ_i denotes the mean of the i^{th} entry (fixed effect), r_k denotes the effect of the k^{th} block (random effect), and ε_{ik} denotes the residual for the observation obtained from the plot assigned to the i^{th} entry in the k^{th} block (random effect or residual).

The residual maximum likelihood estimation procedure was used to generate estimates of variance components and entry means (LS-Means). The statistical comparison was conducted by testing for a difference in LS-Means between DP56113 SPTA maintainer and the control maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method. The same transformations applied during across-site analysis were also utilized for individual-site analyses.

False Discovery Rate Adjustment

The false discovery rate (FDR) method (Benjamini and Hochberg, 1995; Westfall et al., 1999) was used to control for false positive outcomes across all analytes analyzed using linear mixed models. A false positive outcome occurs if the difference in means between two entries is declared significant, when in fact the two means are not different. Since its introduction in the mid-1990s, the FDR approach has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science, and signal/image processing (*e.g.*, Pawitan et al., 2005; Spelman and Bovenhuis, 1998). In the FDR method, the false discovery rate is held at 5% across comparisons of multiple analytes via an adjustment to the P-value and is not inflated by the number of analytes in the comparison. The FDR adjustment of raw P-values was conducted separately for the across-site analysis and each of the individual-site analyses.

Statistical Software and Procedures

Statistical analyses were conducted using SAS software, Version 9.4. SAS PROC MIXED was utilized to fit Models 1 and 2, and to provide LS-Means, 95% confidence intervals, and statistical comparisons. SAS PROC MULTTEST was utilized to provide FDR adjusted P-values. Fisher's exact test was executed via SAS PROC FREQ. All other data processing was conducted in Base SAS.

Interpretation of Statistical Results

For a given analyte, when a statistically significant difference (P-value from mixed model analysis < 0.05, or Fisher's exact test P-value < 0.05) was identified in the across-site analysis, the respective range of individual values from DP56113 SPTA maintainer was compared to a tolerance interval. Tolerance intervals are expected to contain at least 99% of the values for corresponding analytes of the conventional maize population with a 95% confidence level (Hong et al., 2014). The tolerance intervals were derived from Pioneer's accumulated data from non-GM maize lines, which were grown in commercial maize-growing regions in the United States, Canada, and South America between 2003 and 2015. The combined data represent 93 commercial maize lines and 88 unique environments. The selected commercial maize lines represent the non-GM maize population with a history of safe use, and the selected environments (site and year combinations) represent maize growth under a wide range of

environmental conditions (*i.e*. soil texture, temperature, precipitation, and irrigation) and maize maturity group zones.

If the range of DP56113 SPTA maintainer contained individual values outside the tolerance interval, it was then compared to the respective literature range obtained from published literature (Codex Alimentarius Commission, 2013; Cong et al., 2015; ILSI, 2016; Lundry et al., 2013; OECD, 2002; Watson, 1982). Literature ranges compliment tolerance intervals in that they are composed of non-proprietary data from additional non-GM commercial maize lines and growing environments, which are not included in Pioneer's proprietary database.

If the range of DP56113 SPTA maintainer contained individual values outside the literature range, it was then compared to the respective in-study reference range comprised of all individual values across-sites from all non-GM reference maize lines grown in this study. In-study reference data ranges compliment tolerance intervals and literature ranges in that they provide additional context of natural variation specific to the current study.

In cases when a raw P-value indicated a significant difference, but the FDR adjusted P-value was >0.05, it was concluded that the difference was likely a false positive.

In addition, for analytes exhibiting a statistically significant difference (P-value < 0.05) in the across-site mixed model analysis, the results for individual sites were evaluated.

Reported Statistics

The statistical results for transformed data were back-transformed to the original data scale for reporting purposes. For analytes examined using mixed model analysis, the following statistical results were reported: LS-Means, ranges, 95% confidence intervals, FDR-adjusted P-values, and non-adjusted P-values. For the remaining analytes that were not examined using mixed model analysis, the following statistical results were reported: arithmetic means, ranges, and P-value (if Fisher's exact test was conducted). Additionally, three reference ranges (tolerance interval, literature range, and in-study reference range) were provided as available. For fatty acid analytes, LLOQ values were not available on a % total fatty acids basis; therefore, when all sample values were below the LLOQ for a given analyte, mean and range were reported as "<LLOQ".

Table A7-1. Maize Growth Stage Description

Growth Stage	Description
VE	The stage when the plant first emerges from the soil.
V1	The stage when the collar of the first leaf becomes visible.
V2	The stage when the collar of the second leaf becomes visible.
V3	The stage when the collar of the third leaf becomes visible.
V4	The stage when the collar of the fourth leaf becomes visible.
V5	The stage when the collar of the fifth leaf becomes visible.
V6	The stage when the collar of the sixth leaf becomes visible.
V7	The stage when the collar of the seventh leaf becomes visible.
V8	The stage when the collar of the eighth leaf becomes visible.
V9	The stage when the collar of the ninth leaf becomes visible.
V10	The stage when the collar of the tenth leaf becomes visible.
VT	The stage when the last branch of tassel is completely visible.
R1	The stage when silks become visible.
R2	The stage when kernels are white on the outside and resemble a blister in shape.
R3	The stage when kernels are yellow on the outside and the inner fluid is milky white.
R4	The stage when the material within the kernel produces a doughy consistency.
R5	The stage when all or nearly all the kernels are dented or denting.
R6	Typical grain harvest would occur. This stage is regarded as physiological maturity.

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

Appendix 8. Methods and Materials used to Measure Cold, Warm and Diurnal Germination of DP56113 SPTA Maintainer

Experimental Design

Three separate germination tests (warm, cold, and diurnal) were conducted. For a given germination test, 400 seed from each of DP56113 SPTA maintainer, control maize, PH24E maize, PHB00 maize, PH3KP maize, PHCER maize, PHCJP maize, and PHEJW maize were evaluated. The seed from each maize line were arranged into eight individual replicates, with 50 seed per replicate.

Procedures employed to control bias in this study included random placement of each germination replicate and uniform maintenance of environmental conditions across all replicates within each controlled environment.

Germination Tests

The warm germination test simulated emergence under optimal growth conditions, the cold germination test simulated emergence under early planting conditions (ISU Seed Laboratory, 2016), and the diurnal germination test simulated emergence under daily temperature fluctuations (according to ISTA, 2017). For a given germination test, each 50-seed replicate was placed between sheets of moist germination toweling and rolled up with a piece of wax paper wrapped around the moist toweling. The rolls were vertically placed in storage racks.

For the warm germination test, the rolls were transferred to a 24-hour light controlled environment at a continuous setting of 25 °C and 90% relative humidity for 7 days. After 7 days, the number of normal and abnormal germinated seed and the number of hard, fresh, or dead ungerminated seed in each roll were counted.

For the cold germination test, the rolls were transferred to a 24-hour light controlled environment of

10 °C and approximately 90% relative humidity for 7 days, followed by 5 days at a continuous setting of 25 °C and approximately 90% relative humidity.

For the diurnal germination test, the rolls were transferred to a 24-hour light controlled environment of 10 °C and approximately 90% relative humidity for 16 hours followed by a second 24-hour light controlled environment of 25 °C and approximately 90% relative humidity for 8 hours, repeated daily for 10 days. After 10 days, the number of normal and abnormal germinated seed and the number of hard, fresh, or dead ungerminated seed in each roll were counted.

Seed Evaluation

Classification of Germinated and Ungerminated Seed

At the end of each germination test, each seed was classified as either germinated or ungerminated. Descriptions of germination test classifications are provided in Table 14.

Germinated seed were considered viable. Ungerminated seed classified as hard or fresh were further evaluated for viability using a subjective color TZ test (AOSA/SCST, 2010). Ungerminated seed classified as dead were considered non-viable and no further assessments of viability were conducted.

Evaluation of Hard or Fresh Ungerminated Seed Viability

The seed was bisected longitudinally through the embryo; half was placed in a petri dish, and then stained with 0.1% TZ solution and incubated for 1 hour at ambient temperature. After incubation, the seed was evaluated. Any living cells were stained a reddish-pink color by the TZ solution, allowing identification of viable tissues. Any seed with staining patterns indicative of viable tissue in the essential seed structures (*e.g.*, radicle, embryo axis, plumule, and coleoptile) were considered viable. All other TZ-tested seed were considered non-viable.

Statistical Methods

Statistical analyses of germination data were conducted to evaluate the germination rate of seed derived from DP56113 SPTA maintainer compared to the germination rate of seed derived from the control maize. Statistical analyses were conducted separately for each of the three germination tests (warm, cold, and diurnal) using SAS software (Version 9.4).

For a given germination test, GLMM assuming binomial distribution with the “logit” link function was utilized to analyze the data. Maximum likelihood method with Laplace approximation (SAS Institute Inc., 2008; Vonesh, 1996) was utilized to estimate and compare mean germination rates between DP56113 SPTA maintainer and the control maize. A significant difference was established if the P-value was < 0.05. SAS PROC GLIMMIX was utilized to implement GLMM. The “logit” link function, which transformed the parameter π_{ij} on the unit scale into a parameter on the linear predictor scale, denoted as η_{ij}., was expressed as:

$$\eta_{ij} = \text{logit}(\pi_{ij}) = \ln(\frac{\pi_{ij}}{1-\pi_{ij}}),$$

where y_{ij} represent the number of germinated seed in the j^{th} replicate (each replicate contained 50 seed) of the i^{th} entry, j = 1, 2, …, 8; y_{ij} ~ Binomial (n_{ij}, π_{ij}), where n_{ij} denotes the total number of seed in the j^{th} replicate of the i^{th} entry, and π_{ij} denotes the probability of a seed being germinated in the j^{th} replicate of the i^{th} entry.

The GLMM on the linear predictor scale was

$$\eta_{ij} = \mu_i + r_{j(i)},$$

where μ_i denotes the mean response for the i^{th} entry (fixed effect) and $r_{j(i)}$ denotes the effect of the j^{th} replicate within the i^{th} entry (random effect nested within fixed effect). For this model, it was assumed that random effects $r_{j(i)}$ ~ N(0, σ^2_R) were independently and identically distributed of each other.

Reported statistics for each germination test included descriptive statistics (total germination frequency, and range of germination rates for individual replicates, labeled as Frequency and Range, respectively) for DP56113 SPTA maintainer and the control maize, and P-values for statistical comparisons between the two (labeled as P-Value). Estimated mean germination rates and standard errors of the estimates are also provided (labeled as Mean and SE, respectively) for DP56113 SPTA maintainer and the control maize. For each germination test, the range of germination rates for individual replicates across non-GE conventionally bred maize lines (labeled as Reference Range) is provided.

Appendix 9. Methods and Materials used to measure Agronomic Characteristics of DP56113 SPTA Maintainer

Experimental Design

The field portion of this study was conducted during the 2017 growing season at eight sites in commercial maize-growing regions of the United States (two sites in Iowa, one site in Illinois, Indiana, Minnesota, Nebraska, Pennsylvania, and Washington). A randomized complete block design with four blocks was utilized at each site. Each block included DP56113 SPTA maintainer, non-GE near-isoline control maize (referred to as control maize), and four of the following non-GE conventionally bred maize lines: PH12K9, PHWRW, PHB00, PHR1R, PHEJW, PH134G, PH3KP, PH1M3S, PH8JR, PH24E, PHCJP, PH1DCP, PHR62, and PHCER (referred to as reference maize).

Bias in this portion of the study was controlled through the use of non-systematic selection of plot areas within each site, randomization of maize entries within each block, and uniform maintenance treatments across each plot area, with the exception of site RG004IA1 where irrigation was applied to Rows 3-6 of each plot and not the entire trial area.

Field Trial

Planting

Each block contained DP56113 SPTA maintainer, control maize, and four reference maize lines planted in six-row plots at a rate of 30 seeds per row. Each row was 20 ft (6.1 m) in length and 30 in. (76 cm) in width. Each block was separated by an alley of at least 3 ft (0.9 m) in width, and each plot was bordered on either side by one row of maize.

Maintenance Product Applications

At a given site, maintenance products were uniformly applied, as needed, to all plots to minimize weed, insect, and disease pressure (with the exception of at site RG004IA1 where irrigation drip lines were not placed uniformly across the site). Glufosinate-ammonium, glyphosate, ALS inhibitor, 2,4-D, and HPPD inhibitor herbicides were not used post emergence as maintenance pesticides in this study.

Temperature, Rainfall, and Irrigation Records

Daily minimum and maximum temperatures, rainfall totals, and irrigation totals were reported at each site from the planting date to the harvest date.

Agronomic Characteristics Data Collection

The following agronomic characteristics were evaluated from each plot at each site.

Early Stand Count

The total number of emerged plants in Rows 1-4 was determined between the V2 and V4 growth stages.

Time to Flowering

The date when approximately 50% of plants in Rows 1-4 (Rows 3 and 4 only at RG004IA1) had begun shedding pollen was recorded (with the exception of site RG004WA1 for one plot of PHB00 maize and PHEJW maize where abiotic stressors affected the growth and development of some rows and data was collected from the remaining rows that were more representative of the unaffected portions of the plot). These dates were used in subsequent statistical analysis to calculate heat units to flowering.

Pollen Viability (Shape and Color at 0, 30, 60, and 120 Minutes)

When plants in Rows 1-4 were actively shedding pollen, the percentage of non-viable pollen seeds were assessed at four time points by recording the percentage of seeds with collapsed walls and yellow color (Luna et al., 2001).

Plant Height

Plant height was measured in centimeters (with the exception of site RG004IL7 where measurements were collected in inches and converted) from the soil surface to the collar of the flag leaf (base of the tassel) for five individual plants in Rows 1-4 (Rows 3 and 4 only at RG004IA1) at the R4 growth stage.

Lodging

Lodging was evaluated at the R6 growth stage in Rows 1-4 (Rows 3 and 4 only at RG004IA1). Stalk lodging was recorded as the number of plants in each plot with stalks broken below the primary ear. Root lodging was recorded as the number of plants in each plot with stalks leaning approximately 45 degrees or more. A combined lodging score was calculated from stalk and root lodging values.

Final Stand Count

The total number of remaining plants in Rows 1-4 was recorded at the R6 growth stage.

Days to Maturity

The date when 90% of the plants in Rows 1 and/or 2 first reached physiological maturity was recorded.

Ear Count

The number of ears per plant (*i.e.*, ears with kernels) from five plants in Rows 1-4 (Rows 3 and 4 only at RG004IA1) was recorded at the R6 growth stage.

Yield

The seed from Rows 3 and 4 in each plot was harvested at the R6 growth stage. The weight of the seed was recorded in pounds at all sites. Seed weight values from all sites were adjusted to a standardized moisture content and used to calculate yield during subsequent statistical analysis.

Harvest Seed Moisture

The moisture content (%) of harvested seed from Rows 3 and 4 at the R6 growth stage was recorded.

100-Kernel Weight

The total weight (g) of 100 kernels sampled from the pooled seed harvested from Rows 3 and 4 of each plot was determined. 100-kernel weight values were adjusted to a standardized moisture content.

Biotic and Abiotic Observations

Biotic and abiotic observations were taken from Rows 1-4 of each plot. Each plot was evaluated for four observation periods: early vegetative (V2-V5), late vegetative (V7-V9), early reproductive

(R1-R2), and late reproductive (R3-R6) growth stages. Insect damage incidence, plant pathogen incidence, and abiotic stress were evaluated by recording the severity of plant tissue damage caused by each of three insects predominant to the local area, three pathogens predominant to the local area, and three abiotic stressors, respectively. The following ratings were used to evaluate plant damage: "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate symptoms between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death).

Statistical Methods

Statistical analyses were conducted to evaluate and compare agronomic characteristics of DP56113 SPTA maintainer and the control maize.

Processing of Data

Early Stand Count and Final Stand Count

For early stand count and final stand count data, the recorded count value was divided by count area to calculate the number of plants per m^2.

Time to Flowering

For time to pollen shed data, heat units were calculated for each growing day from the recorded planting date to the recorded pollen shed date, and then summed to calculate the total accumulated heat units. Heat units for a given growing day were calculated using the following formula:

((maximum temperature + minimum temperature)/2) – 50 °F

Any temperature values recorded in degrees Celsius would be converted to degrees Fahrenheit for calculation of heat units. If any temperature value was greater than 86 °F, then 86 °F was used as the maximum temperature for that growing day. If any temperature value was less than 50 °F, then 50 °F was used as the minimum temperature for that growing day.

Days to Maturity

For days to maturity data, the number of days was calculated from the recorded planting date to the recorded maturity date.

Plant Height and Ear Count

For plant height and ear count data, the recorded values for five individual plants were used to calculate the plot average.

Lodging

For lodging data, the numbers of root-lodged plants and stalk-lodged plants were summed and then divided by the final stand count to convert to a percentage basis.

Yield

Yield was determined based on the weight of seed collected at typical harvest maturity as follows:

Seed weight was adjusted to 0% moisture content (Seed dry weight):

Seed dry weight (lb) = Seed fresh weight (lb) × (1 - % actual moisture)

Seed dry weight was then adjusted to 15.5% moisture content:

Seed weight at 15.5% moisture (lb) = Seed dry weight (lb) / (1 – 15.5% moisture)

Seed weight at 15.5% moisture was then converted to a yield in bushels per acre (bu/A):

$$\text{Yield (bu/A at 15.5\% moisture)} = \frac{(\text{Seed weight (lb) at 15.5\% moisture}) \times \left(43{,}560 \frac{\text{ft}^2}{\text{A}}\right)}{(\text{plot area (ft2)}) \times \left(56 \frac{\text{lb}}{\text{bu}}\right)}$$

Plot area was calculated by first converting unit of measurement to feet and then using the following formula:

plot area (ft^2) = row length (ft) × row width (ft) × number of rows.

100-Kernel Weight

100-kernel weight for each plot was determined as follows:

Weight of 100 kernels was adjusted to 0% moisture content (100-kernel dry weight):

100-kernel dry weight (g) = 100-kernel fresh weight (g) × (1 - % actual moisture)

100-kernel dry weight was then adjusted to 15.5% moisture content:

100-kernel weight at 15.5% moisture (g) = 100-kernel dry weight (g) / (1 – 15.5% moisture)

Selection of Statistical Method

The following rules were implemented for each agronomic characteristic:

If < 50% of sites had uniform data values for either DP56113 SPTA maintainer or the control maize, and < 50% of all data across sites for each entry were at a uniform value, then an across-site mixed model analysis would be conducted. In addition, if both maize lines had at least two data points at a given site that were not at a uniform value, then an individual-site mixed model analysis would be conducted.

If ≥ 50% of sites had uniform data values for either DP56113 SPTA maintainer or the control maize, and ≥ 50% of sites had uniform data values across both maize lines, then statistical analyses would not be performed.

If the criteria described above were not met, then an across-site analysis using the generalized Cochran-Mantel-Haenszel (CMH) test would be conducted. Individual-site analyses would not be performed.

Across-Site Analysis

Mixed Model Analysis

For a given agronomic characteristic, data were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ Model 1

$\ell_j \sim iid\ N(0, \sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0, \sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Ent\times Site})$, and $\varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$,

where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓ_j denotes the effect of the j^{th} site (random effect), $r_{k(j)}$ denotes the effect of the k^{th} block within the j^{th} site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect), and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} site (random effect or residual). Notation $\sim iid\ N(0, \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a. Subscript *a* represents the corresponding source of variation.

The residual maximum likelihood estimation procedure was utilized to generate estimates of variance components and entry means across sites. The estimated means are known as empirical best linear unbiased estimators (hereafter referred to as LS-Means). The statistical comparison was conducted by testing for a difference in LS-Means between DP56113 SPTA maintainer and the control maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method (Kenward and Roger, 2009). A significant difference was identified if a P-value was < 0.05.

For each agronomic characteristic, goodness-of-fit of the model was assessed in terms of meeting distributional assumptions of normally, independently distributed errors with homogeneous variance. Deviations from assumptions were addressed using an appropriate transformation or a heterogeneous error variance structure.

Generalized CMH Test

The generalized CMH test is more appropriate in the instance where the normality assumption of mixed model analysis cannot be achieved for discrete data. The test was developed specifically for stratified nominal-by-ordinal contingency tables (Agresti, 2002; Koch et al., 1990). It compares entries (a nominal variable) based on their values (recorded on an ordinal scale) while controlling for location (the stratifying variable). Due to the data values being used as the scores in the generalized CMH test, the test's P-value can be directly interpreted as testing for the difference between the arithmetic means of two entries. A significant difference was identified if a P-value was < 0.05.

Individual-Site Analyses

For a given agronomic characteristic, individual sites were analyzed separately using the following linear mixed model:

$y_{ik} = \mu_i + r_k + \varepsilon_{ik}$ Model 2

$r_k \sim iid\ N(0,\sigma^2_{Rep})$ and $\varepsilon_{ik} \sim iid\ N(0, \sigma^2_{Error})$,

where μ_i denotes the mean of the i^{th} entry (fixed effect), r_k denotes the effect of the k^{th} block (random effect), and ε_{ik} denotes the residual for the observation obtained from the plot assigned to the i^{th} entry in the k^{th} block.

The residual maximum likelihood estimation procedure was used to generate estimates of variance components and entry means (LS-Means). The statistical comparison was conducted by testing for difference in LS-Means between DP56113 SPTA maintainer and the control maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method. The same transformations applied during across-site analysis were also utilized for individual-site analysis.

False Discovery Rate Adjustment

The false discovery rate (FDR) method (Benjamini and Hochberg, 1995; Westfall et al., 1999) was used to control for false positive outcomes across all agronomic characteristics analyzed using linear mixed models or generalized CMH tests. A false positive outcome occurs if the difference in means between two entries is declared significant, when in fact the two means are not different. Since the introduction of the FDR approach in the mid-1990s, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science, and signal/image processing (*e.g.,* Pawitan et al., 2005; Spelman and Bovenhuis, 1998). In the FDR method, the false discovery rate is held at 5% across comparisons of multiple agronomic characteristics via an adjustment to the p-value and is not inflated by the number of agronomic characteristics in the comparison. The FDR adjustment of raw P-values was conducted separately for the across-site analysis and each of the individual-site analyses.

Statistical Software and Procedures

Statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA). SAS PROC MIXED was utilized to fit Models 1 and 2, and to provide LS-Means, 95% confidence intervals, and statistical comparisons. SAS PROC FREQ was used to perform the generalized CMH test. SAS PROC MULTTEST was utilized to provide FDR adjusted P-values. All other data processing was generated by Base SAS.

Interpretation of Statistical Results

For a given agronomic characteristic, when a statistically significant difference (P-value < 0.05) was identified in the across-site analysis, the respective range of individual values from DP56113 SPTA maintainer was compared to the in-study reference range comprised of all individual values across-sites from all reference maize lines grown in this study. In cases when a raw P-value indicated a significant difference but the FDR adjusted P-value was >0.05, it was concluded that the difference was likely a false positive. In addition, for agronomic characteristics exhibiting a statistically significant difference (P-value < 0.05) in the across-site analysis, the results for individual sites were evaluated.

Reported Statistics

The statistical results for transformed data were back-transformed to the original data scale for reporting purposes. For agronomic characteristics examined using mixed model analysis, the following statistical results were reported: LS-Means, ranges, 95% confidence intervals, FDR-adjusted P-values, and non-adjusted P-values. For agronomic characteristics examined using CMH test, the following statistical results were reported: arithmetic means, ranges, FDR-adjusted

p-values, and non-adjusted p-values. For agronomic characteristics which were not statistically analyzed, arithmetic means and ranges were reported. Additionally, the in-study reference range was provided for all agronomic characteristics.

Appendix 10. Field Insect and Disease Observations and Methods

Experiment A – 2018 Field Trial Biotic and Abiotic Stressor Measurement

The field portion of this study was conducted during the 2017 growing season at 8 sites in maize-growing regions of the United States (two sites in Iowa, Illinois, and one site in each of Illinois, Indiana, Minnesota, Nebraska, Pennsylvania, and Washington). A randomized complete block design with four blocks was utilized at each site.

Biotic and abiotic observations were taken from each plot. Each plot was evaluated for four observation periods: early vegetative (V2-V5), late vegetative (V7-V9), early reproductive (R1-R2), and late reproductive (R3-R6) growth stages. Insect damage incidence, plant pathogen incidence, and abiotic stress were evaluated by recording the severity of plant tissue damage caused by each of three insects predominant to the local area, three pathogens predominant to the local area, and three abiotic stressors, respectively. The following ratings were used to evaluate plant damage: “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); “moderate” indicates intermediate symptoms between slight and severe; and “severe” indicates symptoms damaging to plant development (*e.g.*, stunting or death). The results for the biotic and abiotic observations are provided in Tables A10-1 to A10-8.

Table A10-1. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004IA1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PH134G Maize	PH8JR Maize	PHCJP Maize	PHR62 Maize
V2-V5 Growth Stages (Evaluated 6/3/2017)							
Insect Damage	AM	None	None	None	None	None	None
	BB	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	CS	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	MPI	Slight-Moderate	Slight	Slight	Slight-Moderate	Slight-Moderate	Slight-Moderate
	WD	Slight	Slight	None-Slight	Slight	Slight	Slight
	WL	Slight-Moderate	Slight	None-Slight	Slight-Moderate	Slight-Moderate	Slight-Moderate
V7-V9 Growth Stages (Evaluated 6/16/2017)							
Insect Damage	AP	None	None	None	None	None	None
	BB	None	None	None-Slight	None-Slight	None-Slight	None
	ECB	None	None-Slight	None	None-Slight	None-Slight	None
Pathogen Stressor	CS	None-Slight	None-Slight	None	None-Slight	None	None-Slight
	ES	None	None	None	None	None	None
	NLS	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	HS	Slight	Slight	Slight	Slight	Slight	Slight
	MPI	None	None	None-Slight	None	None	None
R1-R2 Growth Stages (Evaluated 7/13/2017)							
Insect Damage	FB	None-Slight	None-Slight	None-Slight	None-Slight	None	None
	JP	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None	None-Slight	None-Slight	None-Slight	None	None-Slight
	RSC	None	None	None-Slight	None-Slight	None-Slight	None-Slight
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	SS	None	None	None	None-Slight	None-Slight	None
	WD	None-Slight	None-Slight	None-Slight	None	None	None-Slight
R3-R6 Growth Stages (Evaluated 8/18/2017)							
Insect Damage	CEW	None	None	None-Slight	None-Slight	None	None
	GH	None-Slight	None	None-Slight	Slight	None	None-Slight
	RW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
	RSC	None-Slight	None	None-Slight	None-Moderate	None-Slight	Slight-Moderate
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None	None	None-Slight	None-Slight

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), billbugs weevils (BB), black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), flea beetles (FB), grasshopper (GH), Japanese beetles (JP), and adult rootworms (RW). Pathogen stressors consisted of anthracnose (AN), corn stunt (CS), eyespot (ES), grey leaf spot (GLS), northern leaf spot (NLS), common maize rust (RSC), and stalk rot (SR). Abiotic stressors consisted of drought (DR), heat stress (HS), sun scald (SS), waterlogging (WL), wind damage (WD), and maintenance and non-target pesticide injury (MPI).

Table A10-2. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004IA7

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PH12K9 Maize	PHR1R Maize	PH24E Maize	PHCER Maize
V2-V5 Growth Stages (Evaluated 5/31/2017)							
Insect Damage	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	TH	None	None	None	None	None	None
Pathogen Stressor	BSR	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	CD	None	None	None	None	None	None
	MPI	None	None	None	None	None	None
	SS	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 7/5/2017)							
Insect Damage	AP	None	None	None	None	None	None
	CEW	None	None	None	None	None	None
	JP	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	BSR	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HL	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 7/25/2017)							
Insect Damage	AP	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	JP	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	ES	Slight	Slight	Slight	Slight	Slight	Slight
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
	SMT	None	None	None-Slight	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	Slight-Moderate	None-Moderate	None-Slight	Slight-Moderate	None-Slight	Moderate
R3-R6 Growth Stages (Evaluated 8/23/2017)							
Insect Damage	CEW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	GH	None-Slight	None-Slight	None	None-Slight	None-Slight	None
Pathogen Stressor	GLS	None-Slight	None	Slight	None	None-Slight	None-Slight
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
	SMT	None	None	None	None	None-Slight	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HL	None	None	None	None	None	None
	WD	Slight-Moderate	None-Slight	None-Slight	None-Slight	None	Slight-Moderate

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of aphids (AP), corn ear worm (CEW), European corn borer (ECB), black cutworms (BCW), grasshopper (GH), Japanese beetles (JP), and thrips (TH). Pathogen stressors consisted of bacterial stalk rot (BSR), eyespot (ES), grey leaf spot (GLS), common maize rust (RSC), smut (SMT), and stalk rot (SR). Abiotic stressors consisted of cold (CD), drought (DR), hail (HL), heat stress (HS), sun scald (SS), wind damage (WD), and maintenance and non-target pesticide injury (MPI).

Table A10-3. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004IL7

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PH1M3S Maize	PH24E Maize	PH1DCP Maize	PHR62 Maize
V2-V5 Growth Stages (Evaluated 6/15/2017)							
Insect Damage	BCW	None	None	None	None	None	None
	FAM	None-Slight	None	None-Slight	None	None-Slight	None-Slight
	FB	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	ES	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
	WL	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 7/5/2017)							
Insect Damage	FAM	None	None-Slight	None	None	None	None-Slight
	FB	Slight	Slight	Slight	Slight	Slight	Slight
	JP	None-Slight	None-Slight	Slight	Slight	Slight	None-Slight
Pathogen Stressor	GLS	None	None	None	None	None	None
	RSC	Slight-Moderate	Slight-Moderate	Slight	Slight	Slight	Moderate
	SW	None	None	None	None	None	None
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	SCP	None	None	None	None	None	None
	SS	None-Slight	None-Slight	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/2/2017)							
Insect Damage	FAM	None-Slight	None-Slight	None-Slight	None-Slight	None	None
	FB	None-Slight	None-Slight	None-Slight	Slight	None-Slight	None-Slight
	GH	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None-Slight	None-Slight	Slight	None	None-Slight	None-Slight
	SCP	Slight	Slight	Slight	Slight	Slight	Slight
R3-R6 Growth Stages (Evaluated 9/13/2017)							
Insect Damage	CEW	Slight	Slight	Slight	Slight	Slight	Slight
	GH	Slight	Slight	Slight	Slight	Slight	Slight
	RW	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	AN	None	None	None	None	None	None
	ER	Slight	Slight	None-Slight	None-Slight	Slight	Slight
	SMT	None	None	None-Slight	None	None-Slight	None
Abiotic Stressor	DR	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	SCP	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of corn ear worm (CEW), black cutworms (BCW), fall armyworms (FAM), flea beetles (FB), grasshopper (GH), Japanese beetles (JP), and adult rootworms (RW). Pathogen stressors consisted of anthracnose (AN), ear rot (ER), eyespot (ES), grey leaf spot (GLS), common maize rust (RSC), smut (SMT), and Stewart's wilt (SW). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), soil crusting (SCP), sun scald (SS), waterlogging (WL), and wind damage (WD).

Table A10-4. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004IN2

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PH12K9 Maize	PH8JR Maize	PH1DCP Maize	PHCER Maize
V2-V5 Growth Stages (Evaluated 6/19/2017)							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	WBC	None	None	None	None	None	None
Pathogen Stressor	AN	Slight	Slight	Slight	Slight	Slight	Slight
	GLS	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	MPI	None	None	None	None	None	None
	WD	Slight	Slight	Slight	Slight	Slight	Slight
	WL	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 7/8/2017)							
Insect Damage	ECB	None-Slight	None	None	None	None	None
	JP	None-Slight	None-Slight	None	None-Slight	None-Slight	None-Slight
	SB	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	HLS	None	None-Slight	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	MPI	None	None	None	None	None	None
	WD	Slight-Moderate	Slight	Slight-Moderate	None	None-Slight	Severe
	WL	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/7/2017)							
Insect Damage	AP	Slight-Moderate	Slight-Moderate	Slight-Moderate	None	Slight-Moderate	Slight
	ECB	None	None-Slight	None	None	None-Slight	None-Slight
	FAM	None-Slight	None	None-Slight	None	None-Slight	None-Moderate
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None	None	None	None	None	None
	RSC	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Abiotic Stressor	DR	None	None	None	None	None	None
	MPI	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 9/15/2017)							
Insect Damage	AP	Slight-Moderate	Slight-Moderate	Slight	None	Moderate	Slight-Moderate
	CEW	Moderate	Slight-Moderate	None-Slight	None-Slight	None-Moderate	Slight-Moderate
	ECB	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None
	RSC	Slight	Slight	Slight	Slight-Moderate	Slight-Moderate	Slight
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	MPI	None	None	None	None	None	None
	ND	None-Slight	None-Slight	None-Slight	Slight	None-Slight	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), black cutworms (BCW), corn ear worm (CEW), European corn borer (ECB), fall army worm (FAM), Japanese beetles (JP), sap beetles (SB), and western bean cutworm (WBC). Pathogen stressors consisted of anthracnose (AN), grey leaf spot (GLS), northern leaf blight (NLB), holcus leaf spot (HLS), common maize rust (RSC), and stalk rot (SR). Abiotic stressors consisted of drought (DR), nutrient deficiency (ND), waterlogging (WL), wind damage (WD), and maintenance and non-target pesticide injury (MPI).

Table A10-5. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004MN1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PHB00 Maize	PH3KP Maize	PH1M3S Maize	PH1DCP Maize
V2-V5 Growth Stages (Evaluated 6/5/2017)							
Insect Damage	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	FB	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	BNT	Slight	Slight	Slight	Slight	Slight	Slight
	FR	None	None	None	None	None	None
	HL	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 7/6/2017)							
Insect Damage	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	FB	None	None	None	None	None	None
Pathogen Stressor	NLB	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
	UB	None	Slight	None	None	None	None
Abiotic Stressor	BW	None	None	None	None	None	Slight
	DD	None	None	None	None	None-Slight	None
	WD	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/11/2017)							
Insect Damage	AP	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	NLB	Slight	Slight	Slight	Slight	Slight	Slight
	RSC	Slight	Slight	Slight	None-Slight	Slight	None-Slight
	UB	None	Slight	None	None	None	None
Abiotic Stressor	BW	None	None	None	None	None	Slight
	DD	None	None	None	None	None-Slight	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 9/15/2017)							
Insect Damage	AP	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	Slight	Slight	Slight	Slight	None-Slight	Slight
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	BW	None	None	None	None	None	Slight
	DD	None	None	None	None	None-Slight	None
	WD	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of aphids (AP), European corn borer (ECB), black cutworms (BCW), flea beetles (FB), and adult rootworms (RW). Pathogen stressors consisted of anthracnose (AN), eyespot (ES), grey leaf spot (GLS), northern leaf blight (NLB), unknown blight (UB), common maize rust (RSC), and stalk rot (SR). Abiotic stressors consisted of below normal temperatures (BNT), buggy-whipping (BW), deer damage (DD), hail (HL), frost (FR), and wind damage (WD).

Table A10-6. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004NE1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PH134G Maize	PH3KP Maize	PHCJP Maize	PHCER Maize
V2-V5 Growth Stages (Evaluated 6/8/2017)							
Insect Damage	FB	None	None	None	None	None	None
	JP	None	None	None	None	None	None
	WG	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	NLS	None	None	None	None	None	None
Abiotic Stressor	MPI	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	SCR	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/23/2017)							
Insect Damage	AP	None	None	None	None	None	None
	GH	None	None	None	None	None	None
	SB	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	NLS	None	None	None	None	None	None
Abiotic Stressor	MPI	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 7/28/2017)							
Insect Damage	ECB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	GW	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	HL	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 8/28/2017)							
Insect Damage	AP	None	None	None-Slight	None	None	None
	SM	None-Slight	None-Slight	None-Slight	None-Slight	None	None-Slight
	WBC	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	Slight	None	None	Slight
	GW	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of aphids (AP), European corn borer (ECB), flea beetles (FB), Japanese beetle (JP), grasshopper (GH), sap beetle (SB), spider mites (SM), western bean cutworm (WBC), white grub (WG), and adult rootworms (RW). Pathogen stressors consisted of eyespot (ES), Goss's bacterial wilt (GW), grey leaf spot (GLS), northern leaf blight (NLB), northern leaf spot (NLS), and common maize rust (RSC). Abiotic stressors consisted of hail (HL), nutrient deficiency (ND), soil crusting (SCR), waterlogging (WL), wind damage (WD), and maintenance and non-target pesticide injury (MPI).

Table A10-7. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004PA1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PHWRW Maize	PHR1R Maize	PHEJW Maize	PHR62 Maize
V2-V5 Growth Stages (Evaluated 6/12/2017)							
Insect Damage	ECB	None	None	None	None	None	None
	FB	None	None-Slight	None	None	None	None
	SG	Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
Pathogen Stressor	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	SCR	None	None	None	None	None	None
	WL	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/30/2017)							
Insect Damage	FB	None	None	None	None	None	None
	JP	None	None	None	None	None	None
	LH	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	DR	None-Slight	None	None-Slight	None-Slight	None-Moderate	None-Slight
	HS	None	None	None	None	None	None
	SS	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/4/2017)							
Insect Damage	JP	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
	RW	None	None	None	None	None	None
	SB	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	HS	None	None	None	None	None-Moderate	None-Moderate
	ND	None	None	None	None	None-Moderate	None
	WL	None	None	None	None	None	None
R3-R6 Growth Stages (Evaluated 9/5/2017)							
Insect Damage	CEW	None	None	None	None	None	None
	ECB	None	None-Slight	None	None	None	None
	GH	None	None-Slight	None	None-Slight	None-Slight	None-Slight
Pathogen Stressor	GLS	Moderate	Slight-Moderate	Severe	Slight-Moderate	Moderate	Moderate
	NLB	Slight	Slight	Slight	Slight-Moderate	Slight	Slight-Moderate
	RSC	Moderate	Slight	Moderate	Slight	Slight-Moderate	Slight-Moderate
Abiotic Stressor	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of corn ear worm (CEW), European corn borer (ECB), flea beetles (FB), grasshopper (GH), sap beetles (SB), leafhoppers (LH), Japanese beetles (JP), slugs (SG), and adult rootworms (RW). Pathogen stressors consisted of grey leaf spot (GLS), northern leaf blight (NLB), common maize rust (RSC), and stalk rot (SR). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), soil crusting (SCR), sun scald (SS), wind damage (WD), and waterlogging (WL).

Table A10-8. Biotic and Abiotic Observations of DP56113 SPTA Maintainer at Site RG004WA1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP56113 Maintainer	Control Maize	PHWRW Maize	PHB00 Maize	PHR1R Maize	PHEJW Maize
V2-V5 Growth Stages (Evaluated 6/5/2017)							
Insect Damage	AM	None	None	None	None	None	None
	AP	None	None	None	None	None	None
	TH	None	None	None	None	None	None
Pathogen Stressor	CS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
V7-V9 Growth Stages (Evaluated 6/30/2017)							
Insect Damage	AM	None	None	None	None	None	None
	AP	None	None	None	None	None	None
	TH	None	None	None	None	None	None
Pathogen Stressor	CS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
R1-R2 Growth Stages (Evaluated 8/4/2017)							
Insect Damage	AM	None	None	None	None	None	None
	AP	None-Slight	None-Slight	None-Slight	None	None	None-Slight
	SM	None-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate
Pathogen Stressor	BSR	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
	SMT	None	None	None-Slight	None	Slight	None
Abiotic Stressor	HS	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WL	None-Slight	None	None	None-Moderate	None	None-Moderate
R3-R6 Growth Stages (Evaluated 9/18/2017)							
Insect Damage	AP	None	None	None	None	None	None
	CEW	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
	SM	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate
Pathogen Stressor	BSR	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
	SMT	None	None	None-Slight	None-Slight	Slight	None
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None-Slight	None	None	None-Moderate	None	None-Moderate

Note: A rating of "none" indicates no damage/symptoms observed; "slight" indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); "moderate" indicates intermediate between slight and severe; and "severe" indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), corn ear worm (CEW), thrips (TH), and spider mites (SM). Pathogen stressors consisted of corn stunt (CS), northern leaf blight (NLB), smut (SMT), common maize rust (RSC), bacterial stalk rot (BSR), and Stewart's wilt (SW). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), waterlogging (WL), and wind damage (WD).

Experiment B – DP56113 SPTA Maintainer 2009-2017 Field Insect and Disease Observations

DP56113 SPTA maintainer has been field tested in the United States and Puerto Rico since 2014, as authorized by USDA-APHIS permits and notifications (Appendix 1). For each trial, a survey of the naturally occurring insects and diseases and any unexpected differences in the response of DP56113 SPTA maintainer as compared to the control line were recorded by experienced plant breeders and field staff at least every four weeks. The plant breeders and field staff were familiar with plant pathology and entomology and recorded the severity of any insect or disease in the field. These observations provide a means to determine if DP56113 SPTA maintainer is expected to respond differently from conventional maize lines to insects or diseases in the environment.

A summary of the naturally-occurring insects in the field observations and any unexpected differences seen between DP56113 SPTA maintainer and control lines is presented in Table A10-10. A summary of diseases present in the field observations is presented in Table A10-11.

The following scale was used to evaluate DP56113 SPTA maintainer and control lines:

- Mild – very little disease or insect injury (<10%) visible
- Moderate – noticeable plant tissue damage (10% - 30%)
- Severe – significant plant tissue damage (>30%)

Abiotic stressor field observations were recorded at all United States and Puerto Rico locations and are presented in Table A10-9.

In every case, DP56113 SPTA maintainer did not exhibit any unexpected responses to naturally-occurring insects, diseases or abiotic stressors as compared to the control line.

Table A9-9. Observations of Abiotic Stressors Present and Comparison Between DP56113 SPTA Maintainer and Control Maize

Year	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2016	16-039-106n	CA	Yolo	Nitrogen stress	Moderate	None
		IL	Mason	Slight frost damage occurred evenly across the entire location.	Mild	None
		IA	Louisa	Prescribed drought and low nitrogen stress	Moderate	None
			Polk	Stalk and root lodging due to excessive winds and rain.	Mild	None
				Root and stalk lodging due to heavy rain and high wind events.	Mild	None
				Root lodging due to high winds and heavy rain.	Moderate	None
2017	17-019-105rm	IN	Boone	Wind/Hail	Mild	None
				Drought	Mild	None
		MN	Freeborn	Cold and wet conditions after emergence.	Mild	None
		PA	Lehigh	Drought in some plots	Mild	None
		WA	Grant	Due to a couple of rainfall events, the soil surface developed a crust that some plants struggled to emerge through. Fairly uniformly affecting all plots in entire plot area.	Mild	None
	17-038-103rm-a1	IA	Polk	Lodging from storm and high winds	Mild	None
				Lodging from wind storm	Mild	None
				Lodging from high winds	Mild	None

Table A9-10. Observations of Insects Present and Comparison Between DP56113 SPTA Maintainer and Control Maize

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2014	**14-092-103n**	HI	Kauai	Chinese Rose Beetle, Leafhoppers, Ladybug	Mild	None
				Corn Earworm, Honey Bee, Moth, Spidermites, Lacewing, Leafhopper	Mild	None
				Corn Earworm, Leafhopper, Honey Bee, Spidermites	Mild	None
				Corn Earworm, Spidermites, Honey Bee	Mild	None
				Ladybug, Chinese rose beetle, Leafhopper	Mild	None
				Ladybug, Leafhopper, Honey Bee, Lacewing	Mild	None
				Leafhopper, Corn Earworm	Mild	None
				Spidermites, Corn Earworm, Leafhoppers, Honey Bee	Mild	None
2015	**15-012-104n**	HI	Kauai	Corn Earworm, Leafhopper, Moth	Mild	None
				Lacewing, Spidermites, Leafhopper, Corn Earworm	Mild	None
				Ladybug, Lacewing, Leafhopper	Mild	None
				Leafhopper	Mild	None
				Leafhopper, Corn Earworm	Mild	None
				Moth, Corn Earworm, Spidermites	Mild	None
				Moths	Mild	None
				Spidermites, Lacewing	Mild	None
				Spidermites, Leafhopper, Corn Earworm, Moth	Mild	None

Table A9-10. Observations of Insects Present and Comparison Between DP56113 SPTA Maintainer and Control Maize (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2016	**16-039-106n**	CA	Yolo	Corn earworm (Helicoverpa zea)	Moderate	None
				Mites (Acari)	Moderate - Severe	None
		IL	Mason	Japanese beetle (Popillia japonica)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Moderate	None
			McDonough	Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Northern corn rootworm (Diabrotica barberi)	Mild	None
		IA	Louisa	European corn borer (Ostrinia nubilalis)	Mild	None
				Other - Nematodes	Moderate	None
			Polk	Mild - Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
2017	**16-293-101rm-a1**	PR	Guayama	Aphids (Aphididae)	Mild	None
				Armyworms (Spodoptera spp.)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Corn sap beetle (Carpophilus spp.)	Mild	None
				Leafhopper (Cicadellidae)	Mild	None
				Other - Spidermite	Mild	None

Table A9-10. Observations of Insects Present and Comparison Between DP56113 SPTA Maintainer and Control Maize (continued)

Year	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-019-105rm	IN	Boone	Armyworms (Spodoptera spp.)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild	None
				Aphids (Aphididae)	Moderate	None
		NE	York	Grasshoppers (Orthoptera)	Mild	None
				Two-spotted spider mite (Tetranychus urticae)	Mild	None
		PA	Lehigh	Corn earworm (Helicoverpa zea)	Mild	None
				European corn borer (Ostrinia nubilalis)	Mild	None
				Grasshoppers (Orthoptera)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Other - Slugs	Mild	None
		WA	Grant	Aphids (Aphididae)	Mild	None
				Armyworms (Spodoptera spp.)	Mild	None
				Corn earworm (Helicoverpa zea)	Mild - Moderate	None
				Mites (Acari)	Mild - Moderate	None
	17-038-103rm-a1	IA	Polk	Aphids (Aphididae)	Mild	None
				Japanese beetle (Popillia japonica)	Mild	None
				Western corn rootworm (Diabrotica virgifera virgifera)	Mild	None
	17-263-106rm	HI	Kauai	Chinese rose beetle (Adoretus sinicus)	Mild	None
2019	19-091-106rm	NE	York	No data*		
	19-105-102r	NE	York			

*Planted in June or not yet planted, no data taken yet.

Table A9-11. Observations of Diseases Present and Comparison Between DP56113 SPTA Maintainer and Control Maize

Year	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2014	**14-092-103n**	HI	Kauai	Common Rust	Mild	None
				Virus like	Mild	None
2015	**15-012-104n**	HI	Kauai	Common Rust	Mild	None
2016	**16-039-106n**	CA	Yolo	Common smut (Ustilago maydis)	Mild	None
		IL	Mason	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
			McDonough	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
		IA	Polk	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
2017	**16-293-101rm-a1**	PR	Guayama	Other - Bacteria	Mild	None
				Other - Fungus	Mild	None

Table A9-11. Observations of Diseases Present and Comparison Between DP56113 SPTA Maintainer and Control Maize (continued)

Year	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-019-105rm	IN	Boone	Common rust (Puccinia sorghi)	Mild - Moderate	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
		MN	Freeborn	Common rust (Puccinia sorghi)	Mild	None
				Corn leaf blight (Pleosporaceae)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
		PA	Lehigh	Common rust (Puccinia sorghi)	Mild - Moderate	None
				Northern corn leaf blight (Exserohilum turcicum)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Moderate	None
		NE	York	Common rust (Puccinia sorghi)	Mild	None
				Gray leaf spot (Cercospora zeae-maydis)	Mild	None
				Gray spot (Pyricularia spp.)	Mild	None
	17-038-103rm-a1	IA	Polk	Common rust (Puccinia sorghi)	Mild	None
				Common smut (Ustilago maydis)	Mild	None
2019	19-091-106rm	NE	York	No data*		
	19-105-102r	NE	York			

*Planted in June or not yet planted, no data taken yet.

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