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**Petition for Determination of Nonregulated Status for
Enhanced Grain Yield Potential and Glufosinate-ammonium Resistant
DP202216 Maize**

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Certification

The undersigned submits this petition under 7 CFR §340.6 to request that the administrator make a determination that the article, DP-202216-6 maize (DP202216) not be regulated under 7 CFR §340.

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



5/31/19

Date

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Abbreviations, Acronyms, and Definitions

AACC	American Association of Cereal Chemists
ADF	Acid Detergent Fiber
AOAC	Association of Analytical Chemists
AOCS	American Oil Chemists' Society
ASB-14	Amidosulfobetaine-14
<i>attB1</i>	Bacteriophage lambda integrase recombination site
<i>attB2</i>	Bacteriophage lambda integrase recombination site
<i>attB3</i>	Bacteriophage lambda integrase recombination site
<i>attB4</i>	Bacteriophage lambda integrase recombination site
B73	Inbred maize line
BAPNA	Benzoyl-DL-arginine-p-nitroanilide hydrochloride
BLASTP	Basic Local Alignment Search Tool for Proteins
bp	Base pair
bu/a	Bushels per acre
BW	Body Weight
BWA	Burrows-Wheeler Aligner
°C	Degrees Celsius
CA	Canada
CDP	Chemiluminescent Disodium 2-chloro-5-(4-methoxy Spiro(1,2-dioxetane-3,2'-(5-chlorotricyclo(3.3.1.1 ^{3,7})decan))-4-yl)-1-phenyl phosphate
CEA	Composition, Expression, Agronomic
CI	Confidence Interval
cm	Centimeters
CMH	Cochran-Mantel-Haenszel
<i>colE1 ori</i>	<i>Escherichia Coli</i> origin of replication
<i>cos</i>	Cohesive ends from lambda bacteriophage DNA
<i>ctl</i>	Central control operon region from bacteria
DDE	Daily Dietary Exposure
DDG	Distillers Dried Grains
DEEM	Dietary Exposure Evaluation Model
DI	Deionized
DIG	Digoxigenin
DM	Dry matter
DNA	Deoxyribonucleic acid
DP202216	Maize event DP-202216-6
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EIA	U.S. Energy Information Administration

Abbreviations, Acronyms, and Definitions, (continued)

<i>E</i> -value	Expected value
ELISA	Enzyme-Linked Immunosorbent Assay
FAME	Fatty Acid Methyl Ester
FASTA	FAST-All similarity search program
FCID	Food Commodity Intake Database
FDR	False Discovery Rate
fl oz/A	Fluid ounces per acre
Flp	Flippase
FRT1	Flippase recombinase target site
FRT87	Flippase recombinase target site
FW	Fresh weight
g	Grams
gal/A	Gallons per acre
GC/FID	Gas Chromatography/Flame Ionization Detection
GE	Genetically engineered
GLP	Good Laboratory Practice
<i>gos2</i>	<i>Zea mays</i> translation initiation factor
HCL	Hydrochloric Acid
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectroscopy
in	Inches
kb	Kilobases
kDa	KiloDaltons
kg	Kilogram
kg/ha	Kilograms per hectare
L/ha	Liters per hectare
LB	Left border
lb/A	Pounds per acre
LD-50	Lethal dose 50 percent
LDS/DTT	Lithium Dodecyl Sulfate/Dithiothreitol
LLOQ	Lower limit of quantification
<i>loxP</i>	Bacteriophage P1 recombination site
[]	[] transcription factor
mg	Milligram
ml	Milliliter
mM	Millimolar
MMT	Million metric tonnes
MOE	Margin of Exposure

Abbreviations, Acronyms, and Definitions, (continued)

<i>mo-pat</i>	Maize-optimized phosphinothricin acetyltransferase gene
MS/MS	Tandem Mass Spectrometry
N	Number
NA	Not Applicable
ND	Not Detectable
NDF	Neutral Detergent Fiber
<i>Nco I</i>	<i>Nocardia corallina</i> derived restriction enzyme
ng	Nanograms
NGS	Next Generation Sequencing
NHANES	National Health and Nutrition Examination Survey
nm	Nanometers
non-GE	Non-genetically engineered
OD	Optical Density
OECD	Organization for Economic Co-operation and Development
ORF	Open Reading Frame
<i>oriT</i>	Origin of transfer region from bacteria
<i>oriV</i>	Origin of replication region from bacteria
<i>pat</i>	Phosphinothricin acetyltransferase gene
PAT	Phosphinothricin acetyltransferase protein
PBST	Phosphate Buffered Saline and Polysorbate 20
PCR	Polymerase Chain Reaction
PHP40099	Plasmid 40099
<i>pinII</i>	<i>Solanum tuberosum</i> (potato) proteinase inhibitor II gene
P-value	Probability value
RB	Right border
R1	Maize reproductive growth stage
R4	Maize reproductive growth stage
R6	Maize reproductive growth stage
SbS	Southern by Sequence
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SGF	Simulated Gastric Fluid
<i>spc</i>	Spectinomycin resistance gene
SSC	Saline Sodium Citrate
TBHQ	Tert-Butylhydroquinone
T-DNA	Transfer DNA
<i>tetR</i>	Tetracycline resistance regulation gene from bacteria
<i>tetA</i>	Tetracycline resistance gene from bacteria
TIU	Trypsin Inhibitor Units
<i>trfA</i>	Trans-acting replication gene from bacteria

Abbreviations, Acronyms, Definitions (continued)

μg	Micro gram
<i>ubiZM1</i>	<i>Zea mays</i> ubiquitin gene 1
UPLC	Ultra-Performance Liquid Chromatography
UTR	Untranslated region
UV	Ultraviolet
V	Volts
V6	Maize vegetative growth stage
V9	Maize vegetative growth stage
<i>virD1</i>	Virulence gene from <i>Agrobacterium tumefaciens</i>
<i>virC1</i>	Virulence gene from <i>Agrobacterium tumefaciens</i>
<i>virC2</i>	Virulence gene from <i>Agrobacterium tumefaciens</i>
<i>virG</i>	Virulence gene from <i>Agrobacterium tumefaciens</i>
<i>virB</i>	Virulence operon region from <i>Agrobacterium tumefaciens</i>
WWEIA	What We Eat in America
χ^2	Chi-square
<i>zm-gos2</i>	<i>Zea mays</i> translation initiation factor <i>gos 2</i> gene
<i>zmm28</i>	<i>zmm 28</i> gene
ZMM28	ZMM 28 protein

Summary

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting a Petition for Determination of Nonregulated Status for enhanced grain yield potential and glufosinate-ammonium herbicide resistant maize event DP-202216-6, hereafter referred to as DP202216 maize. Pioneer requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that DP202216 maize, DP202216 maize progeny, and any crosses of DP202216 maize with other nonregulated maize no longer be considered regulated articles under 7 CFR §340.

DP202216 maize was developed by Pioneer using genetic engineering techniques to increase and extend the expression of the maize *zmm28* gene relative to the native *zmm28* gene expression. Both the introduced and native *zmm28* genes encode the ZMM28 protein, a [] transcription factor. The increased and extended expression of the ZMM28 protein results in plants with enhanced grain yield potential via []. DP202216 maize also contains the phosphinothricin acetyltransferase (PAT) protein, which confers resistance to the herbicidal active ingredient glufosinate-ammonium at current labeled rates. The PAT protein present in DP202216 maize is identical to the corresponding protein found in previously authorized events across several different crops that are currently in commercial use.

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DP202216 maize was generated using *Agrobacterium*-mediated transformation with plasmid PHP40099 containing the *zmm28* and *mo-pat* gene cassettes. Molecular characterization of DP202216 maize by Southern blot analysis and a Next Generation Sequencing (NGS) method known as Southern-by-Sequence (SbS™ technology, hereafter referred to as SbS) confirmed that a single, intact PHP40099 DNA fragment was inserted into the genome. Segregation analysis of DP202216 maize confirmed the Mendelian inheritance of the *zmm28* and *mo-pat* genes. Bioinformatics analysis of open reading frames (ORFs) identified no allergenicity or toxicity concerns regarding the identified translated ORFs at the DP202216 maize insertion site.

The potential for allergenicity and toxicity of DP202216 maize was evaluated by examining the allergenic potential of maize as a crop and by assessing the allergenic and toxic potential of the ZMM28 and PAT proteins. Maize is not a common allergenic food and the modification of DP202216 maize is not expected to alter the allergenic potential of maize. The ZMM28 protein expressed in DP202216 maize is a native maize protein with a deduced amino acid sequence that is identical to ZMM28 protein expressed in conventional, non-genetically engineered (non-GE) maize lines. As it is a native maize protein, the ZMM28 protein has a history of safe use as described herein. Expression levels of ZMM28 were measured in edible maize tissues and exposure calculations were performed for humans and livestock. Exposure to the ZMM28

protein via consumption of DP202216 maize grain and forage is low and is not expected to pose a risk to humans or livestock.

The PAT protein in DP202216 maize is identical to the PAT protein found in previously authorized events across several different crops that are currently in commercial use (USDA-APHIS, 2001; USDA-APHIS, 2005; USDA-APHIS, 2013). The PAT protein has been extensively reviewed in numerous preceding regulatory submissions and no evidence of acute toxicity, glycosylation, or identity to known allergens or toxins have previously been identified. The level of expression of the PAT protein in DP202216 maize does not significantly raise exposure of humans or animals when compared against exposure through consumption of previously authorized genetically engineered maize containing the PAT protein.

Extensive nutrient composition analyses of grain and forage (70 total analytes) were conducted to compare the composition of DP202216 maize to that of a control maize line (defined as non-GE, near-isoline in this petition) and 16 conventional (defined as a non-GE hybrid used in commercial production in this petition) maize varieties. These analyses were used to evaluate any changes in the levels of key nutrients, anti-nutrients and secondary metabolites. Based on the results of the compositional evaluation, the grain and forage of DP202216 maize is comparable to conventional maize. Use of DP202216 maize is not expected to result in any significant impact on raw or processed maize commodities.

Comprehensive agronomic performance assessments for DP202216 maize were conducted in replicated field studies at a total of 12 locations in the United States and Canada. The following characteristics were measured: early stand count, days to flowering, height, lodging, final stand count, days to maturity, pollen viability, kernels per ear (calculated from kernel rows per ear and kernels per row), 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 during the growing season. Seed germination and dormancy data were also collected in laboratory experiments. Analysis of agronomic data showed no statistically significant differences between DP202216 maize and control maize lines, with the exception of enhanced yield potential in multi-year broad acreage trials, indicating the agronomic comparability of DP202216 maize to conventional maize. In addition, DP202216 maize has been field tested over 9 years in the United States and Puerto Rico. All releases in the United States have occurred under field permits and notifications granted by USDA - APHIS. All field trials of DP202216 maize were observed for naturally occurring insects or diseases, and no unexpected differences between DP202216 maize and control maize were observed. Together, these data support the conclusion that DP202216 maize is unlikely to pose a greater plant pest risk than conventional maize.

The potential environmental impact of the introduction of DP202216 maize considered three primary areas: the potential for DP202216 maize to become weedy or invasive; the potential for gene flow to sexually compatible wild relatives; and the potential impacts of the introduced PAT protein. Analyses indicated that DP202216 maize was comparable to conventional maize with respect to the nutrient composition and agronomic characteristics measured, with the exception of expected enhanced yield potential. In general, maize does not possess weedy characteristics and is not considered a weedy or invasive species. Therefore, DP202216 maize does not exhibit characteristics that would indicate it is any more likely than conventional maize to become a weed or plant pest. The potential for gene flow examined maize pollination biology and the hybridization potential and geographic overlap of maize wild relatives. While maize does possess some pollination characteristics favorable to gene flow, the distribution of wild relative populations is limited in the United States. Therefore, it is unlikely that the inserted DNA in DP202216 maize would be introgressed into wild relative populations.

The data and information contained herein supports the conclusion that DP202216 maize does not present a plant pest risk and is not otherwise deleterious to the environment. Therefore, Pioneer requests that APHIS grant the request for a determination of nonregulated status for DP202216 maize, DP202216 maize progeny, and any crosses of DP202216 maize with other nonregulated maize.

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

I. Rationale for the Development of DP202216 Maize

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

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

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting data for genetically engineered enhanced grain yield potential and glufosinate-ammonium resistant DP-202216-6 (DP202216) maize and requests a determination from USDA-APHIS that event DP202216, 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 DP202216 Maize

Higher grain yield has historically been achieved through conventional breeding and optimization of crop management practices. Certain phenotypic characteristics are associated with increased maize grain yield (for example, decreased tassel size, change in leaf angle, increased kernel number and kernel weight, delayed senescence, and a longer period of grain fill during plant growth (Duvick, 2005; Echarte et al., 2013; Rajcan and Tollenaar, 1999). By selecting for desired plant phenotypes, conventional breeding approaches have made incremental improvements in maize grain yield and have altered the expression of endogenous maize genes and genetics over time. Using modern biotechnology tools to alter the expression of targeted maize genes that are known to play a role in certain phenotypic characteristics associated with positive grain yield complements the selection of genes through breeding.

DP202216 maize was genetically engineered to increase and extend the expression of the *zmm28* gene relative to the native *zmm28* gene expression. Both the introduced and native *zmm28* genes encode the ZMM28 protein, a [] transcription factor. The increased and extended expression of the ZMM28 protein results in maize plants with enhanced grain yield potential []

[] (Appendix 14). The PAT protein confers resistance to glufosinate ammonium, the active ingredient in phosphinothricin herbicides (CERA - ILSI Research Foundation, 2016).

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Maize has multiple downstream uses for feed, fuel, and food that are significant for the global supply of this crop. The introduction of enhanced yield potential and herbicide-resistant DP202216 maize is intended to help growers keep pace with increasing maize demand globally.

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

DP202216 maize has been field tested in the United States and Puerto Rico over 9 years in more than 100 separate plantings as authorized by the USDA-APHIS permits and notifications (Appendix 1).

A voluntary safety and nutritional assessment of DP202216 maize was submitted to the FDA's Center for Food Safety and Applied Nutrition (FDA CFSAN) in 2018.

Pioneer is committed to robust product stewardship prior to launch and continuing through product discontinuation. Pioneer is a member of Excellence Through Stewardship®. Pioneer products are commercialized in accordance with ETS Product Launch Policy Stewardship Guidance and in compliance with the Pioneer 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 United States and global supply. In 2017, 14.6 billion bushels of maize were produced in the United States from approximately 90.2 million planted acres, valued at \$47.5 billion (USDA-NASS, 2018b; USDA-NASS, 2018a). The United States is a major global exporter of maize at approximately 41% of the total trade market (USDA-FAS, 2018a). The largest maize United States export markets in 2016-2017 were Mexico, Japan, Colombia, Peru, and South Korea (NCGA, 2018a; USGC, 2018). Exports accounted for 11% of the maize produced in 2017 and those exports were shipped to more than 70 countries (NCGA, 2018b; USGC, 2018).

A significant portion of maize cultivated in the United States is genetically engineered. In 2018, 90% of maize grown in the United States was genetically engineered; insect resistant varieties accounted for approximately 82% of all maize acreage, which includes the percentage of insect resistant traits as well as stacked varieties (USDA-ERS, 2018). Over the past decade, maize yields and overall production have increased, in part due to improvements in seed varieties and agronomic production practices (USDA-ERS, 2017).

Maize Processing for Feed, Fuel, and Food Uses

Maize grain requires processing for some 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 (OECD, 2002). 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 (OECD, 2002). Products from the dry milling process include flour, meal, germ meal, oil, beverage and fuel ethanol, distillers dried solubles, flaking grits, hominy feed, and grits (OECD, 2002). 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 distiller dried grains or solubles (OECD, 2002).

Feed Use of Maize

The largest proportion, 33%, of maize produced in the United States is used for animal feed (NCGA, 2018b).

Of the maize grain that is used for feed, the greatest percentage is consumed by poultry, followed by beef cattle, pork, and dairy cattle (NCGA, 2018a). 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 (OECD, 2002). 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 (OECD, 2002). When ears are shelled to remove the grain, remnant cobs can also be used in animal feed (OECD, 2002). Maize grain can be fed to animals with minimal processing and can be fed whole, rolled, ground, or steam flaked (OECD, 2002). Rolled or

ground grain is fed to swine and poultry (OECD, 2002). 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 distillers dried grains/solubles (DDG) or corn gluten feed that is used as animal feed for 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).

Fuel Use of Maize

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

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 2017, 460 million bushels of United States maize went to the production of high-fructose corn syrup as an end product (NCGA, 2018a). Maize produced in the United States in 2017 was also used for production of starch (6 MMT), sweeteners (10 MMT), cereal/food (5 MMT), and beverage alcohol (4 MMT) (NCGA, 2018a).

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

II. The Biology of Maize

II-A. Maize as a Crop

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

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

The subsequent breeding of DP202216 maize 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, [] was used as the recipient line to produce DP202216 maize. Line [] was chosen because it is receptive to transformation and is also an elite line (*i.e.*, Pioneer proprietary line used for commercial products).

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III. Method of Development of DP202216 Maize

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

DP202216 maize was created by *Agrobacterium*-mediated transformation with plasmid PHP40099 (Figure 3; Table 2). The inserted transfer DNA (T-DNA) region from plasmid PHP40099 (Figure 4, Table 3) contains two gene cassettes. Creation of transformation events and resultant plants from PHP40099 occurred in Johnston, Iowa, United States.

The first gene cassette (*zmm28* gene cassette) increases and extends the expression of the *zmm28* gene relative to the native *zmm28* gene expression. Both the introduced and native *zmm28* genes encode the ZMM28 protein, a [] transcription factor. The increased and extended expression of the ZMM28 protein results in maize plants with enhanced grain yield potential. The ZMM28 protein is 251 amino acids in length and has a molecular weight of approximately 28 kDa. Expression of the *zmm28* gene is controlled by the promoter region from the *Zea mays* translation initiation factor *gos2* (*zm-gos2*) gene (de Pater et al., 1992) along with the intron region from the *Zea mays* ubiquitin gene 1 (*ubiZM1*) (Christensen et al., 1992). Transcription of the *zmm28* gene is terminated by the terminator region from the potato (*Solanum tuberosum*) proteinase inhibitor II (*pinII*) gene (An et al., 1989; Keil et al., 1986).

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The second gene cassette (*mo-pat* gene cassette) contains a maize-optimized version of the phosphinothricin acetyl transferase gene (*mo-pat*) from *Streptomyces viridochromogenes* (Wohlleben et al., 1988). The *mo-pat* gene expresses the phosphinothricin acetyl transferase (PAT) enzyme that confers resistance to phosphinothricin. The PAT protein is 183 amino acids in length and has a molecular weight of approximately 21 kDa. Expression of the *mo-pat* gene is controlled by the promoter region from the *ubiZM1* gene, including the 5' untranslated region (UTR) and intron (Christensen et al., 1992). The terminator for the *mo-pat* gene is a second copy of the *pinII* terminator (An et al., 1989; Keil et al., 1986).

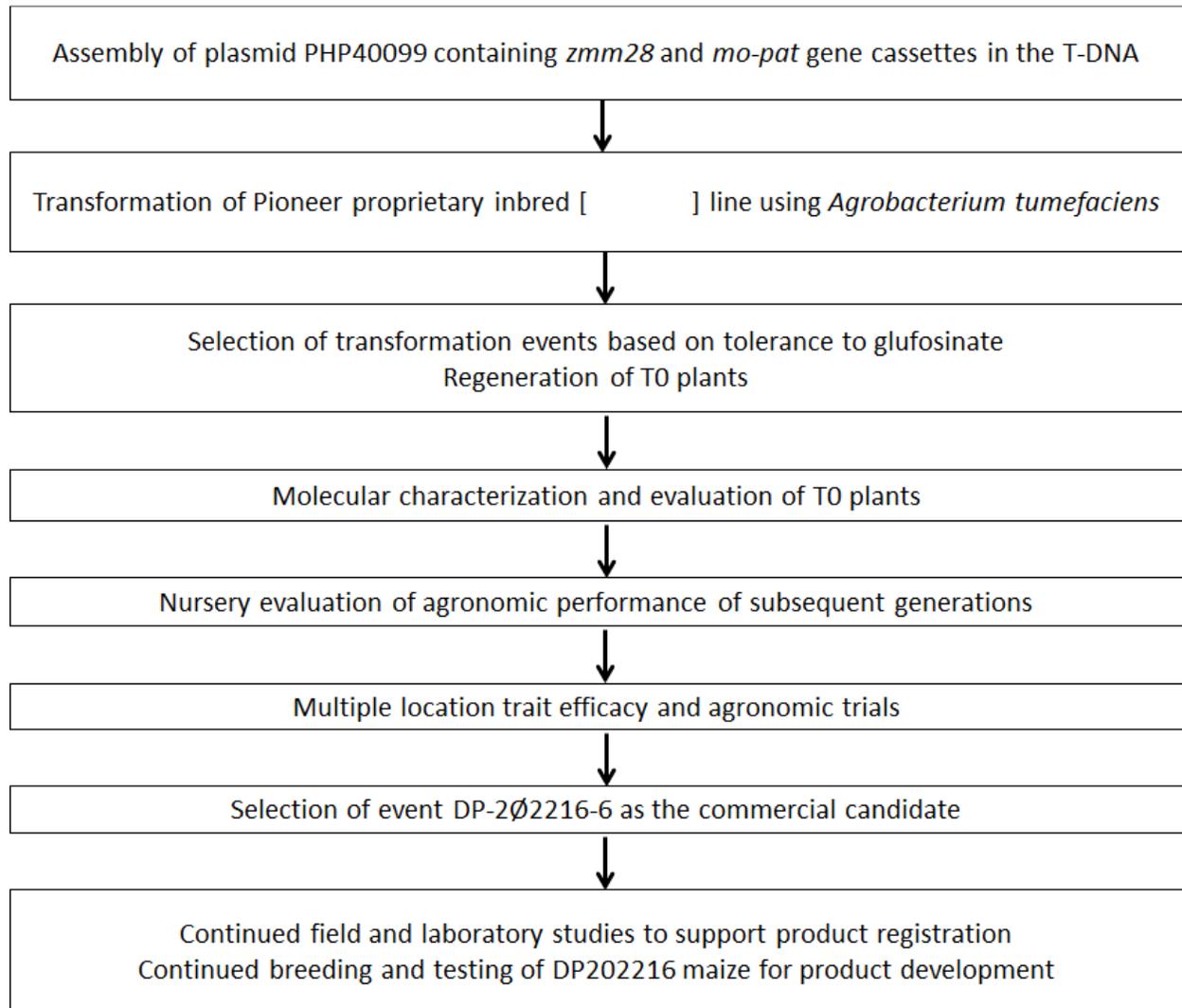
The PHP40099 T-DNA contains two flippase (Flp) recombinase target sites, FRT1 and FRT87 (Proteau et al., 1986; Tao et al., 2007, respectively), as well as two *loxP* (Dale and Ow, 1990) and four *attB* recombination sites, *attB1* and *attB2* (Hartley et al., 2000; Katzen, 2007) and *attB3* and *attB4* (Cheo et al., 2004). The presence of these sites alone does not cause any recombination. To function, these sites need a specific recombinase enzyme that is not naturally present in plants (Cox, 1988; Dale and Ow, 1990; Thorpe and Smith, 1998).

Pioneer proprietary inbred line [] was transformed with plasmid PHP40099 to produce DP202216 maize. Immature maize embryos were harvested from a surface-sterilized ear of [] maize approximately 8-11 days after pollination and inoculated with *Agrobacterium tumefaciens* strain JTLBA4404 containing plasmid PHP40099 (Zhao et al., 2001). *Agrobacterium tumefaciens* strain JTLBA4404 is a disarmed strain that does not contain tumor-inducing factors;

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however, with the inclusion of plasmid PHP40099, the strain will contain 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*. Transformed 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 PHP40099 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)



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Figure 1. Schematic Diagram of the Development of DP202216 Maize

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Figure 2. Breeding Diagram for DP202216 Maize

The breeding steps to produce the generations used for characterization, assessment, and the development of commercial lines are shown schematically. Pioneer proprietary inbred [] was used for transformation to produce DP202216 maize. Pioneer proprietary inbred [] was used in crossing and backcrossing steps.

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Table 1. Generations and Comparators used for Analysis of DP202216 Maize

Analysis	Seed Generation(s) Used	Comparators
Copy Numbers, Integrity, and Backbone by SbS	[]	[]
Integrity and Stability by Southern Blot	[]	[]
Composition and Expression Analysis	[]	[]
Mendelian Inheritance	[]	[]

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III-B. Selection of Comparators for DP202216 Maize

For the characterization of DP202216 maize, Pioneer proprietary maize [] hybrid [] and inbred lines [] were used as experimental controls (Table 1). The control lines selected are non-genetically engineered (non-GE) and represent the genetics of the maize lines used to produce the DP202216 maize generations used in analysis (Figure 2).

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In addition, non-genetically engineered Pioneer™ Brand maize hybrid lines (*i.e.*, reference lines), were used to obtain tolerance intervals for compositional analyses. These maize hybrids were chosen to represent a wide range of non-genetically engineered varieties that would normally be planted commercially. These tolerance intervals represent the normal range of variation of the maize crop for compositional analytes and further helped to determine the comparability of DP202216 maize to conventional maize.

IV. Donor Genes and Regulatory Sequences

IV-A. DNA Used in Transformation

DP202216 maize was produced by *Agrobacterium tumefaciens*-mediated transformation with plasmid PHP40099. (Figure 3), which contains the *zmm28* and *mo-pat* expression cassettes (Figure 4). A summary of the genetic elements and their position in plasmid PHP40099 and the T-DNA region is given in Tables 2 and 3, respectively.

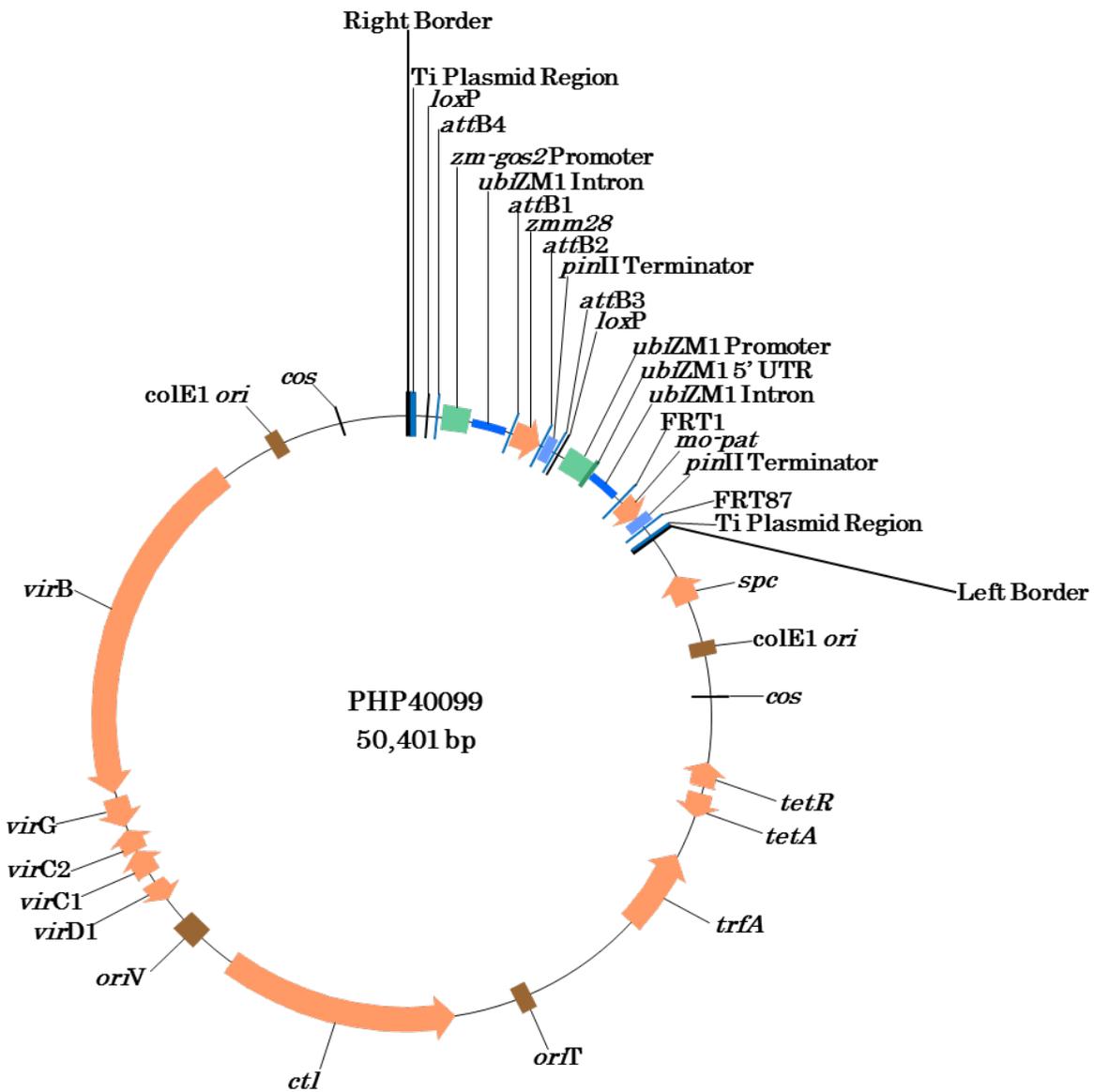


Figure 3: Map of Plasmid PHP40099

Schematic diagram of plasmid PHP40099 indicating the *zmm28* and *mo-pat* genes with regulatory elements. The T-DNA region flanked by the Right Border and the Left Border was inserted into the maize genome during *Agrobacterium*-mediated transformation to produce DP202216 maize. The size of plasmid PHP40099 is 50,401 bp. A description of the genetic elements in plasmid PHP40099 is provided in Table 2.

Table 2. Description of Genetic Elements in Plasmid PHP40099

Region	Location on Plasmid (bp to bp)	Genetic Element	Size (bp)	Description
T-DNA	1 – 7,470		7,470	See Table 3 for information on the elements in this region
Plasmid Construct	7,471 – 32,356	Includes Elements Below	24,886	DNA from various sources for plasmid construction and plasmid replication
	8,646 – 9,434 (complementary)	<i>spc</i>	789	Spectinomycin resistance gene from bacteria (Fling et al., 1985)
	10,557 – 10,926 (complementary)	<i>colE1 ori</i>	370	Origin of replication region from <i>Escherichia coli</i> (Tomizawa et al., 1977)
	12,022 – 12,035	<i>cos</i>	14	Cohesive ends from lambda bacteriophage DNA (Komari et al., 1996)
	13,740 – 14,390 (complementary)	<i>tetR</i>	651	Tetracycline resistance regulation gene from bacteria (Komari et al., 1996)
	14,496 – 15,695	<i>tetA</i>	1,200	Tetracycline resistance gene from bacteria (Komari et al., 1996)
	16,968 – 18,116 (complementary)	<i>trfA</i>	1,149	Trans-acting replication gene from bacteria (Komari et al., 1996)
	21,930 – 22,041	<i>oriT</i>	112	Origin of transfer region from bacteria (Komari et al., 1996)
	23,881 – 30,151 (complementary)	<i>ctl</i>	6,271	Central control operon region from bacteria (Komari et al., 1996)
	31,159 – 31,869 (complementary)	<i>oriV</i>	711	Origin of replication region from bacteria (Komari et al., 1996)
Ti Plasmid Backbone	32,357 – 47,173	Includes Elements Below	14,817	Virulence (<i>vir</i>) gene and intergenic regions from the <i>Agrobacterium tumefaciens</i> Ti plasmid (Komari et al., 1996)
	32,670 – 33,113 (complementary)	<i>virD1</i>	444	Virulence gene from <i>Agrobacterium tumefaciens</i> important for T-DNA insertion into genome
	33,382 – 34,076	<i>virC1</i>	695	Virulence gene from <i>Agrobacterium tumefaciens</i> important for T-DNA insertion into genome
	34,079 – 34,687	<i>virC2</i>	609	Virulence gene from <i>Agrobacterium tumefaciens</i> important for T-DNA insertion into genome
	34,798 – 35,601 (complementary)	<i>virG</i>	804	Virulence gene from <i>Agrobacterium tumefaciens</i> important for T-DNA insertion into genome
	35,733 – 45,168 (complementary)	<i>virB</i>	9,436	Virulence operon region from <i>Agrobacterium tumefaciens</i> important for T-DNA insertion into genome
Plasmid Construct	47,174 – 50,401	Includes Elements Below	3,228	DNA from various sources for plasmid construction and plasmid replication
	47,469 – 47,838 (complementary)	<i>colE1 ori</i>	370	Origin of replication region from <i>Escherichia coli</i> (Tomizawa et al., 1977)
	48,931 – 48,944	<i>cos</i>	14	Cohesive ends from lambda bacteriophage DNA (Komari et al., 1996)

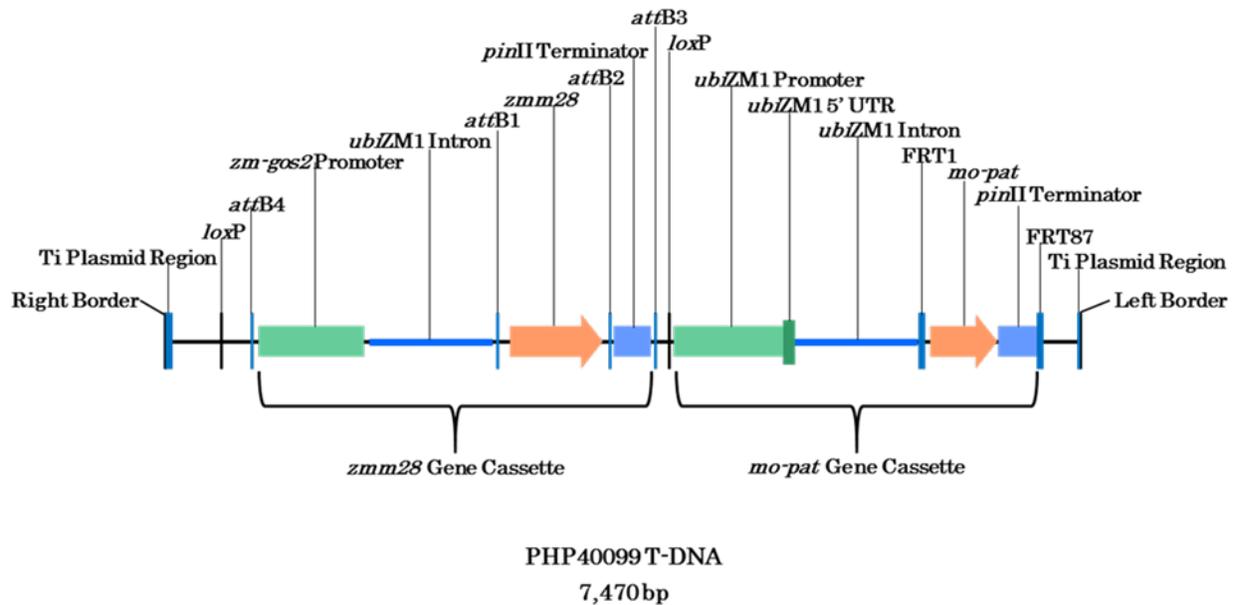


Figure 4: Map of T-DNA Region of PHP40099

Schematic diagram of the PHP40099 T-DNA indicating the *zmm28* and *mo-pat* gene cassettes. The T-DNA was inserted into the maize genome by *Agrobacterium*-mediated transformation to produce DP202216 maize. The size of the T-DNA is 7,470 bp. A complete description of the genetic elements in the T-DNA region of plasmid PHP40099 is provided in Table 3.

Table 3. Description of Genetic Elements in T-DNA Region of Plasmid PHP40099

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 <i>Agrobacterium tumefaciens</i> Ti plasmid (Komari et al., 1996)
	26 – 177	Ti Plasmid Region	152	Sequence from the <i>Agrobacterium tumefaciens</i> Ti plasmid (Komari et al., 1996)
	178 – 435	Intervening Sequence	258	DNA sequence used for cloning
	436 – 469	<i>loxP</i>	34	Bacteriophage P1 recombination site recognized by Cre recombinase(Dale and Ow, 1990)
	470 – 698	Intervening Sequence	229	DNA sequence used for cloning
	699 – 719	<i>attB4</i>	21	Bacteriophage lambda integrase recombination site (Cheo et al., 2004)
	720 – 753	Intervening Sequence	34	DNA sequence used for cloning
zmm28 Gene Cassette	754 – 1,613	<i>zm-gos2</i> Promoter	860	Promoter region from the <i>Zea mays</i> translation initiation factor <i>gos2</i> gene (de Pater et al., 1992)
	1,614 – 1,654	Intervening Sequence	41	DNA sequence used for cloning
	1,655 – 2,667	<i>ubiZM1</i> Intron	1,013	Intron region from the <i>Zea mays</i> ubiquitin gene 1 (Christensen et al., 1992)
	2,668 – 2,707	Intervening Sequence	40	DNA sequence used for cloning
	2,708 – 2,731	<i>attB1</i>	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning system (Hartley et al., 2000; Katzen, 2007)
	2,732 – 2,748	Intervening Sequence	17	DNA sequence used for cloning
	2,749 – 3,605	<i>zmm28</i>	857	[]-domain transcription factor gene region from <i>Zea mays</i> including 5' and 3' untranslated regions (UTR) [] as described below: 5' UTR at bp 2,749-2,808 (60 bp long) Coding sequence at bp 2,809-3,564 (756 bp long) 3' UTR at bp 3,565-3,605 (41 bp long)
	3,606 – 3,621	Intervening Sequence	16	DNA sequence used for cloning
	3,622 – 3,645	<i>attB2</i>	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning system (Hartley et al., 2000; Katzen, 2007)
	3,646 – 3,659	Intervening Sequence	14	DNA sequence used for cloning
	3,660 – 3,967	<i>pinII</i> Terminator	308	Terminator region from the <i>Solanum tuberosum</i> (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)

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Table 3. Description of Genetic Elements in T-DNA Region of Plasmid PHP40099 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	3,968 – 3,997	Intervening Sequence	30	DNA sequence used for cloning
	3,998 – 4,018	<i>attB3</i>	21	Bacteriophage lambda integrase recombination site (Cheo et al., 2004)
	4,019 – 4,091	Intervening Sequence	73	DNA sequence used for cloning
	4,092 – 4,125	<i>loxP</i>	34	Bacteriophage P1 recombination site recognized by Cre recombinase (Dale and Ow, 1990)
	4,126 – 4,144	Intervening Sequence	19	DNA sequence used for cloning
<i>mo-pat</i> Gene Cassette	4,145 – 5,044	<i>ubiZM1</i> Promoter	900	Promoter region from the <i>Zea mays</i> ubiquitin gene 1 (Christensen et al., 1992)
	5,045 – 5,127	<i>ubiZM1</i> 5' UTR	83	5' untranslated region from the <i>Zea mays</i> ubiquitin gene 1 (Christensen et al., 1992)
	5,128 – 6,140	<i>ubiZM1</i> Intron	1,013	Intron region from the <i>Zea mays</i> ubiquitin gene 1 (Christensen et al., 1992)
	6,141 – 6,168	Intervening Sequence	28	DNA sequence used for cloning
	6,169 – 6,216	FRT1	48	Flippase recombination target site from <i>Saccharomyces cerevisiae</i> (Proteau et al., 1986)
	6,217 – 6,242	Intervening Sequence	26	DNA sequence used for cloning
	6,243 – 6,794	<i>mo-pat</i>	552	Maize-optimized phosphinothricin acetyltransferase gene from <i>Streptomyces viridochromogenes</i> (Wohlleben et al., 1988)
	6,795 – 6,801	Intervening Sequence	7	DNA sequence used for cloning
	6,802 – 7,112	<i>pinII</i> Terminator	311	Terminator region from the <i>Solanum tuberosum</i> (potato) proteinase inhibitor II gene (An et al., 1989; Keil et al., 1986)
	7,113 – 7,133	Intervening Sequence	21	DNA sequence used for cloning
	7,134 – 7,181	FRT87	48	Modified flippase recombination target site derived from <i>Saccharomyces cerevisiae</i> (Tao et al., 2007)
	7,182 – 7,388	Intervening Sequence	207	DNA sequence used for cloning
	7,389 – 7,445	Ti Plasmid Region	57	Sequence from the <i>Agrobacterium tumefaciens</i> Ti plasmid (Komari et al., 1996)
	7,446 – 7,470	Left Border (LB)	25	T-DNA Left Border from the <i>Agrobacterium tumefaciens</i> Ti plasmid (Komari et al., 1996)

IV-B. Identity and Source of the *zmm28* and *mo-pat* Gene Cassettes in PHP40099

DP202216 maize was produced by *Agrobacterium*-mediated transformation with a T-DNA fragment (Figure 4) that was isolated from plasmid PHP40099 (Figure 3). A summary of the genetic elements and their position in the PHP40099 T-DNA transformation fragment are listed in Table 3.

***Zea mays*: donor of the *zmm28* gene**

Class: Liliopsida, Monocotyledones
Order: Cyperales
Family: Poaceae (Gramineae)
Genus: *Zea*
Species: *Z. mays* L.

According to the OECD, maize is the world's third leading cereal crop, following wheat and rice. It is grown as a commercial crop in over 25 countries worldwide. Field maize has been grown for 8,000 years in Mexico and Central America and for 500 years in Europe (OECD, 2002). Maize is cross-pollinated, and until about 1925 mainly open pollinated varieties were grown. Hybrid maize is the main variety currently used in production (OECD, 2002). Worldwide production of maize was about 1033 million tons in 2017 (FAO, 2012; USDA-FAS, 2018b).

***Streptomyces viridochromogenes*: donor of the *mo-pat* gene**

Class: Actinobacteria (high G+C Gram-positive bacteria)
Order: Actinomycetales Family:
Streptomycetaceae
Genus: *Streptomyces*
Species: *S. viridochromogenes*
Strain: Tü494

Streptomyces viridochromogenes is a common soil bacterium that is not considered pathogenic to humans or animals (OECD, 2007) and produces the tripeptide phosphinothricyl-L-alanyl-L-alanine, which was developed as a non-selective herbicide. The *mo-pat* gene, encoding the phosphinothricin acetyl transferase (PAT), confers resistance to the phosphinothricin herbicide application (OECD, 1999).

Other Donor Organisms

Potato (*Solanum tuberosum*), was used as a source for the regulatory sequence of the *pinII* terminator that is not expressed in the transformed plant. *Z. mays* is the donor of the *zm-gos2* and *ubiZM1* promoters and intron regulatory regions.

V. Genetic Characterization of DP202216 Maize

V-A. Molecular Analysis Overview

Molecular characterization of genetically engineered 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. DP202216 maize plants were characterized by a Next Generation Sequencing (NGS) method known as Southern-by-Sequencing (SbS™ technology, hereafter referred to as SbS) method to determine the number of insertions within the plant genome, insertion integrity, and to confirm the absence of plasmid backbone sequences. Southern blot analysis was performed to confirm stable genetic inheritance of the inserted *zmm28* and *mo-pat* cassettes.

Based on the SbS analysis described below, it was determined that a single, intact PHP40099 T-DNA was inserted into the genome of DP202216 maize and that no sequences from the backbone of plasmid PHP40099 were inserted. In addition, Southern blot analysis across five breeding generations confirmed the stable genetic inheritance of the DNA insertion in DP202216 maize.

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

SbS identifies inserted DNA within the plant genome (Zastrow-Hayes et al., 2015). The SbS technique utilizes capture probes homologous to the transformation plasmid to isolate genomic DNA that hybridizes to the probe sequences. Captured DNA is then sequenced using a Next Generation Sequencing (NGS) procedure and the results are analyzed using bioinformatics tools.

During the analysis, junction reads are identified as those sequence reads where part of the read shows exact homology to the plasmid DNA sequence while the rest of the read does not match the contiguous plasmid. Junctions may occur between inserted DNA and genomic DNA, or between insertions of two plasmid DNA sequences that are not contiguous in the transformation plasmid.

Multiple sequence reads are generated of each junction and these reads are compiled into a consensus sequence for the junction. By compiling a large number of unique sequencing reads and comparing them to the transformation plasmid and control maize genome, unique junctions due to inserted DNA are identified. A unique junction is defined as one in which the plasmid-derived sequence and the adjacent sequence are the same across multiple reads, although the overall length of the multiple reads for that junction will vary due to the sequencing process. The number of unique junctions is related to the number of plasmid insertions present in the 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 either the presence of additional plasmid insertion(s) or rearrangement(s) of the inserted DNA.

Absence of any junctions indicates there are no detectable insertions within the genome. A schematic diagram of the SbS process is presented in Figure 5.

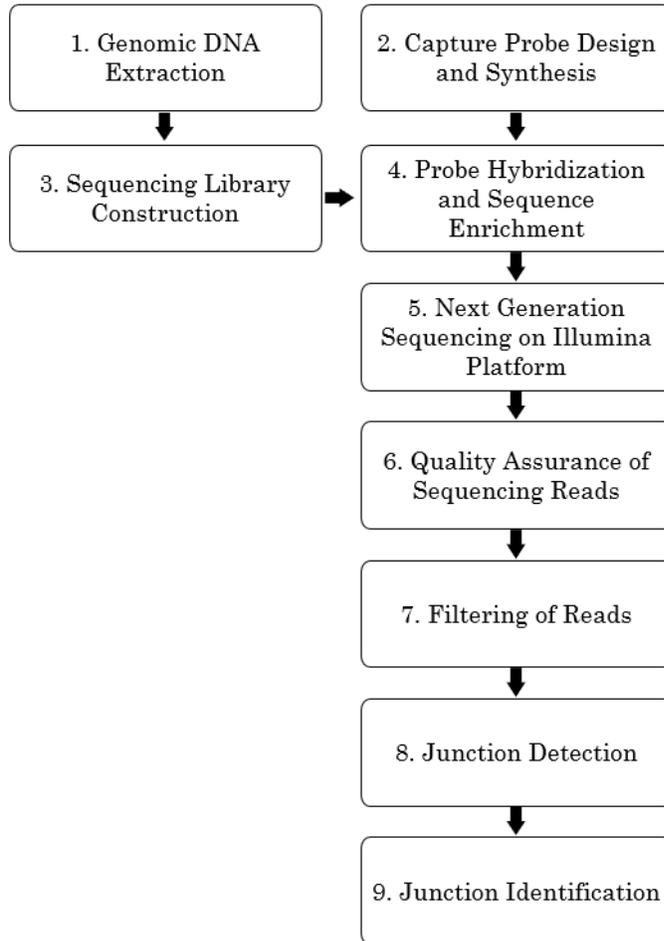


Figure 5. Southern by Sequencing (SbS) Process Flow Diagram

SbS using full-coverage probes comprising the entire sequence of the PHP40099 transformation plasmid was conducted on eight plants from the [] generation of DP202216 maize. A negative control sample ([] maize genomic DNA) and a positive control sample ([] maize genomic DNA spiked with PHP40099 plasmid DNA at a level corresponding to one copy of PHP40099 per copy of the maize genome) were also analyzed by SbS.

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Genomic DNA isolated from DP202216 [] maize and [] control maize plant leaves was analyzed by polymerase chain reaction (PCR) to confirm the presence or absence of the *zmm28* and *mo-pat* genes. Genomic DNA from eight DP202216 maize plants and one control maize plant leaves was also tested with an event-specific assay for the DP202216 insertion. Six of eight DP202216 maize plants tested were PCR positive and confirmed to contain the inserted

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PHP40099 T-DNA (positive plants, Table 4). The remaining two DP202216 maize plants were shown to be PCR negative for the insertion (negative plants; Table 4). The control maize plant was negative for all PCR assays, indicating it did not contain the DP202216 insert.

Results of the SbS analysis showed that two unique junctions were present and consistent in all six plants that were PCR positive for the DP202216 insert. The SbS results for one representative plant are presented in Figure 6. Results for the five remaining DP202216 PCR positive plants are provided in Appendix 2. The 5' junction for all six plants started with base pair (bp) 23 of the PHP40099 T-DNA within the Right Border, and the insertion ended with the 3' junction at bp 7,458 of the T-DNA within the Left Border. These results indicate minor truncations of the T-DNA borders in DP202216 maize. Right Border and Left Border termini deletions often occur in *Agrobacterium*-mediated transformation, as described Kim et al (Kim et al., 2007). The junction locations were identical across all six plants, indicating that the DP202216 DNA insertion is consistent and stable across the [] generation of DP202216 maize.

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SbS analysis of DP202216 maize did not identify junctions between non-contiguous regions of the PHP40099 T-DNA. This indicates that there are no detectable rearrangements or truncations in the inserted DNA, other than the Right Border and Left Border truncations noted above. The number of sequence reads at the 5' and 3' junctions for each plant is provided in Table 4. There were no additional junctions between the PHP40099 plasmid sequence and the maize genome in the DP202216 PCR positive plants, indicating that there are no additional plasmid-derived insertions present in DP202216 maize.

Using the SbS results, a schematic diagram of the DP202216 insertion was developed and is provided in Figure 7.

Several genetic elements in the PHP40099 T-DNA (Figure 4) are derived from maize and homologous elements in the genome of the [] control and PCR negative plants and will be captured by the full-coverage probes used in the SbS analysis. Therefore, the [] control and PCR negative plants will have sequencing reads of maize endogenous elements (*zm-gos2* and *ubiZM1* promoters, *ubiZM1* 5'UTR, *ubiZM1* intron, and *zmm28*) in the SbS results.

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SbS results for the control maize plant and the positive control sample are presented in Figures 8 and 9, respectively. Sequencing reads were detected in the 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 result from the capture and sequencing of these genetic elements in their normal context within the [] maize genome. Variation in coverage of the endogenous elements is due to sequence variation between the [] control maize and the maize varieties from which the genetic elements in PHP40099 were derived. Junctions were not detected between plasmid sequences and the maize genomic

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sequences, indicating there are no PHP40099 plasmid DNA insertions in the control maize, and that the sequence reads were solely due to the endogenous genetic elements.

SbS analysis for the positive control sample resulted in sequence coverage across the entire length of the PHP40099 plasmid (Figure 9). This demonstrates that the SbS analysis utilizing the full-coverage probe library is sensitive enough to detect PHP40099 plasmid sequences at a concentration equivalent to one copy of PHP40099 per copy of the maize genome. Junctions were not detected between plasmid and maize genomic sequences, indicating that the sequence reads were due to the spiked-in plasmid, or to the endogenous maize genetic elements as detected in the [] control maize.

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The two DP202216 maize plants that were PCR negative for the DP202216 insertion were analyzed by SbS and results are shown in Appendix 2 (figures A1-1 and A1-2). While sequence reads were detected in the two negative plants, the coverage of the reads matches the reads in the control maize, indicating the reads are due to endogenous maize sequences. No junctions were detected between the PHP40099 plasmid sequence and maize genome sequence in the PCR negative plants, indicating they did not contain any insertions derived from PHP40099.

There were no junctions identified between maize genomic sequences and the backbone sequence of the PHP40099 plasmid in any of the plants analyzed, demonstrating that no plasmid backbone sequences were incorporated into DP202216 maize.

SbS analysis of the [] generation of DP202216 maize demonstrates that there is a single, intact insertion of the PHP40099 T-DNA in DP202216 maize and that no additional insertions are present in its genome.

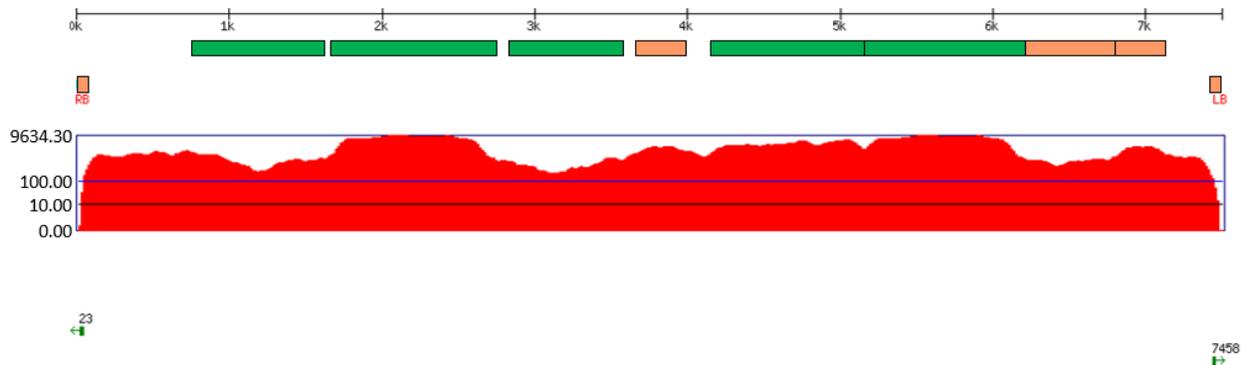
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Table 4. PCR Results and SbS Junction Reads of DP202216 Maize Plants

Plant ID	DP202216 DNA Insertion ¹	Unique Reads at 5' Junction ²	Supporting Reads at 5' Junction ³	Unique Reads at 3' Junction ⁴	Supporting Reads at 3' Junction ⁵
335728647	+	19	457	16	383
335728648	+	22	479	24	422
335728649	—	0	0	0	0
335728650	—	0	0	0	0
335728651	+	25	618	25	416
335728652	+	20	467	14	201
335728653	+	23	740	27	549
335728654	+	19	411	29	535

1. The presence of the DP202216 DNA insertion is based on event-specific and *zmm28* and *mo-pat* gene-specific PCR results.
2. Unique reads supporting the location of the 5' genomic junction of the DP202216 DNA insertion at bp 23 of the PHP40099 T-DNA. Multiple identical NGS supporting reads are condensed into each unique read.
3. Total number of reads across the 5' junction of the DP202216 insertion.
4. Unique reads supporting the location of the 3' genomic junction of the DP202216 DNA insertion at bp 7,458 of the PHP40099 T-DNA. Multiple identical NGS supporting reads are condensed into each unique read.
5. Total number of reads across the 3' junction of the DP202216 insertion.

A.



B.

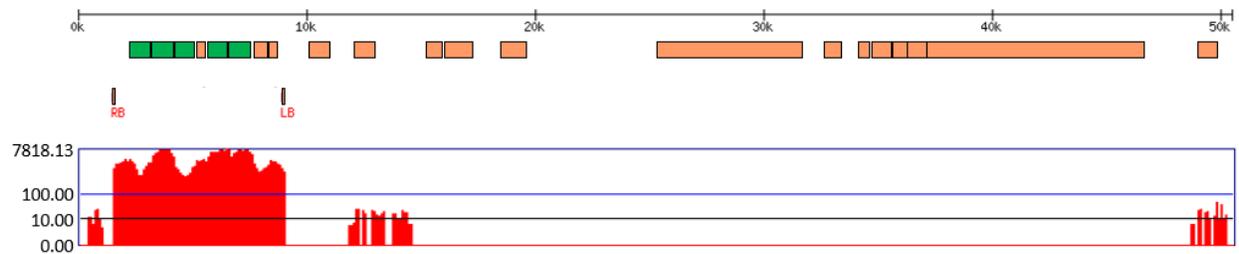


Figure 6. SbS Analysis for a Representative DP202216 Maize Plant

SbS results for a representative [] generation DP202216 maize plant (ID 335728647 in Table 4) that was confirmed to contain the PHP40099 T-DNA insertion. The red coverage graph shows the number of individual NGS reads aligned at each point on the construct using a log scale. Green bars above the coverage graph indicates endogenous genetic elements derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Green arrows in the Junctions section show the two genome-plasmid sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the intact T-DNA sequence. The insertion comprises bp 23 to 7,458 of the PHP40099 T-DNA shown in Figure 4. The presence of only two junctions when aligned to the T-DNA sequence demonstrates the presence of a single PHP40099 T-DNA in the DP202216 maize genome. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the T-DNA region near the left of the coverage graph; however, for clarity the junctions identified in Panel A are not shown in this view. The absence of any other junctions to the PHP40099 sequence indicates that there are no additional insertions or PHP40099 backbone sequence present in DP202216 maize.

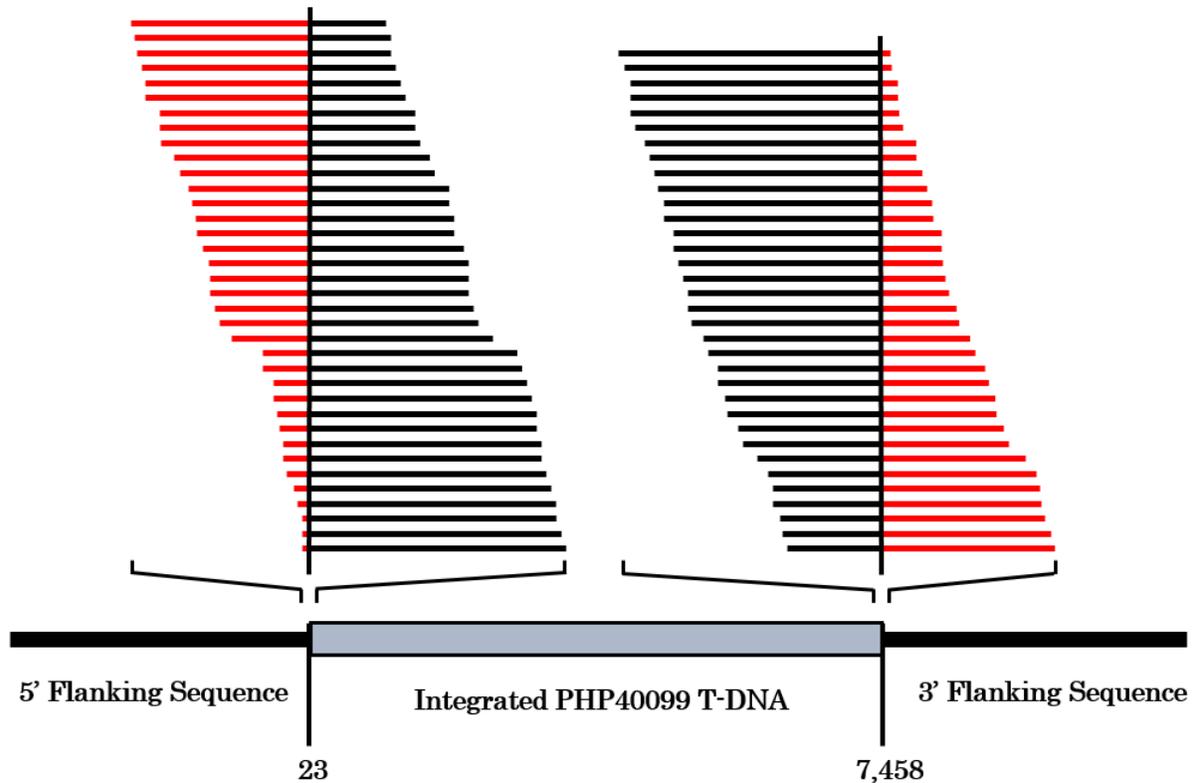
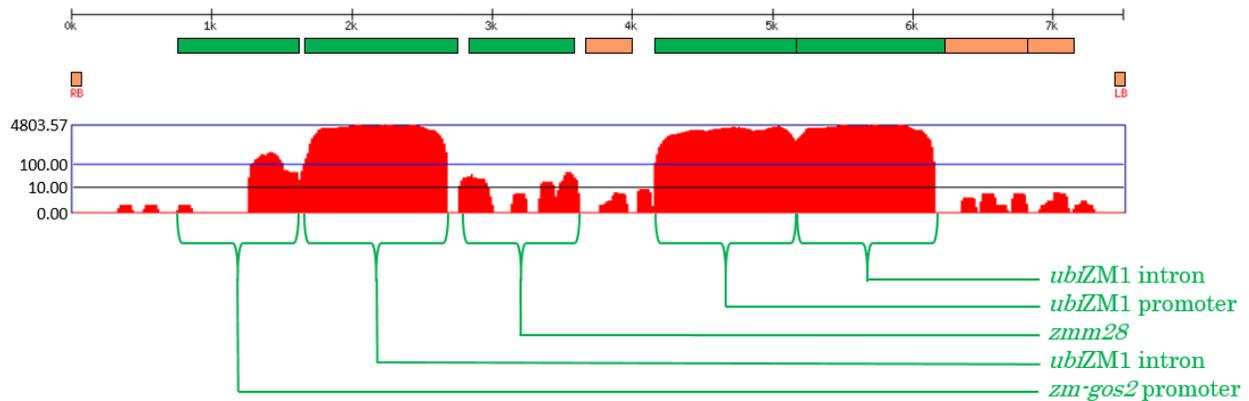


Figure 7: Map of the Insertion in DP202216 Maize

Schematic map of the PHP40099 T-DNA insertion in DP202216 maize based on the SbS analysis. The flanking maize genomic regions are represented by the horizontal black bars. A single copy of the PHP40099 T-DNA, shown by the gray box, is integrated into the maize genome. The numbers below the map indicate the bp location of the junction nucleotide as compared to the sequence of the PHP40099 T-DNA (Figure 4). Representative individual sequencing reads across the junctions are shown as stacked lines above each junction; red indicates genomic flanking sequence and black indicates T-DNA sequence within each individual read. Vertical lines show the locations of the junctions.

A.



B.

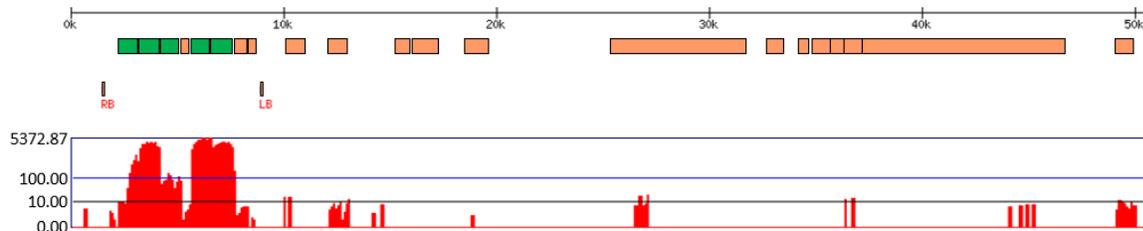


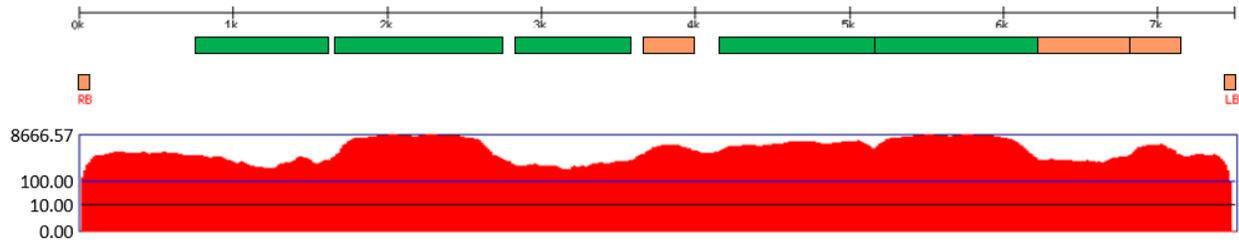
Figure 8. Sbs Analysis for Control Maize

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 PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** Sbs results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Coverage above background level (35x) was obtained only for regions derived from maize endogenous elements (labeled in green font). 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 PHP40099. No junctions were detected between plasmid sequences and the maize genomic sequences, indicating that there are no DNA insertions in the wild-type maize, and the sequence reads are solely due to the endogenous elements present in the [] genome. **B)** Sbs results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the same endogenous elements as in Panel A. The absence of any junctions to the PHP40099 sequence indicates that there are no insertions or PHP40099 backbone sequence present in the [] control maize.

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A.



B.

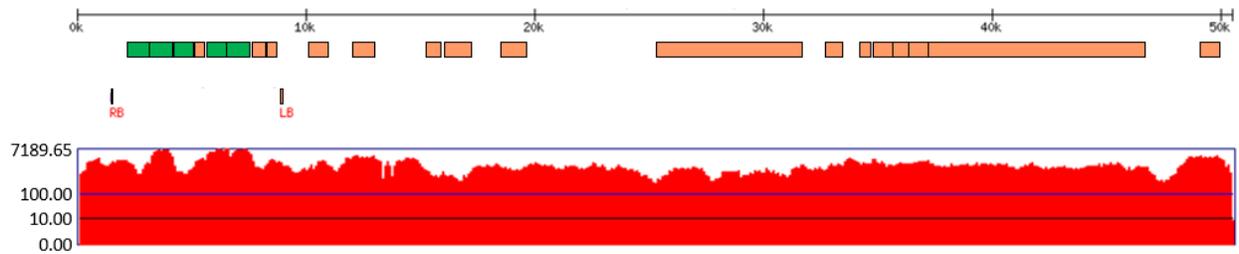


Figure 9. SbS Analysis for the Positive Control Sample

The positive control sample consisted of [] maize genomic DNA spiked with PHP40099 plasmid DNA at a level corresponding to one copy of PHP40099 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 log scale. Green bars above the coverage graph indicates endogenous genetic elements derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Coverage was obtained for the entire T-DNA, indicating efficient capture by the probe library of sequence from the PHP40099 plasmid added to maize genomic DNA. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained across the full length of the plasmid, again indicating successful capture of PHP40099 sequences by the SbS probe library.

V-C. Southern Blot Analysis of DP202216 Maize

The DNA insertion in DP202216 maize was characterized by Southern blot analysis to evaluate the integrity and stability of the inserted *zmm28* and *mo-pat* gene cassettes.

Restriction sites used in Southern analysis are indicated on the schematic map of the PHP40099 plasmid (Figure 10). All probes used for the analysis are indicated on the schematic map of the PHP40099 T-DNA region (Figure 11) and outlined in Table 5.

Southern blot analysis was conducted on five generations of DP202216 maize to demonstrate the inserted DNA remained stable across multiple generations. Genomic DNA samples from individual plants of the [] generations of DP202216 maize and control maize lines [] were analyzed by digestion with restriction enzyme *Nco* I. The *Nco* I-digested genomic DNA samples were hybridized with the *zmm28* and *mo-pat* gene probes to demonstrate that the DP202216 insertion is intact and remained stable across all five generations of DP202216 maize. The presence of equivalent bands from hybridization with the *zmm28* and *mo-pat* probes within all five generations analyzed confirmed that the DP202216 maize insertion is stable and equivalent across multiple generations.

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Restriction enzyme *Nco* I was selected to verify the stability of the DP202216 insertion between the five generations [] of DP202216 maize plants. *Nco* I was selected because there is a single *Nco* I restriction site within the PHP40099 T-DNA (Figure 11), which provides a means to uniquely identify the event, as additional sites would be in the adjacent flanking genomic DNA (Figure 12). Genomic DNA samples from the five generations of DP202216 maize and control maize plants were digested with *Nco* I and hybridized with the *zmm28* and *mo-pat* gene probes for Southern analysis. The *zmm28* and *mo-pat* hybridization patterns exhibited event-specific bands unique to the DP202216 insertion, and thus provided a means of verification that the DP202216 insertion remained intact across the five generations during breeding. Plasmid PHP40099 was added to control maize DNA, digested with *Nco* I, and included on the blot to verify successful probe hybridization.

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Since the *zmm28* gene is derived from the maize genome, the *zmm28* gene probe is expected to hybridize to its endogenous gene and genes with homologous sequence found in the maize genome. Thus, additional hybridization bands in all the DP202216 and control maize samples were expected. The [] generation DP202216 samples are of [] control maize genetic background; whereas those of the [] generations are of [] control maize background. Endogenous bands of DP202216 maize at the [] generations align with those of the [] control maize; whereas endogenous bands of DP202216 maize at [] generations match those in the [] control maize line. These endogenous bands are identified in Table 6 by asterisks (*) and gray shading.

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Hybridization of the *zmm28* probe to *Nco* I-digested genomic DNA resulted in a consistent band of approximately 10,000 bp in all five generations of DP202216 maize (Table 6, Figure 13). In addition to the insertion-derived band, there were multiple endogenous bands observed across the DP202216 maize and control maize samples, of approximately: 12,000 bp, 8,500 bp, 6,500 bp, 5,500 bp, 4,500 bp, 4,200 bp, 3,800 bp, 3,400 bp, 3,000 bp, 2,500 bp, 2,200 bp, 1,800 bp and 1,400 bp (Table 6, Figure 13). These bands can be attributed to hybridization of the probe to endogenous sequences in the maize genome that are homologous to the *zmm28* probe. Endogenous bands in the DP202216 maize samples are the same as the control maize line of their respective genetic backgrounds. This result confirmed that the 5' border fragment, containing the *zmm28* gene in the DP202216 insertion, is intact and stable across the five generations of DP202216 maize. The plasmid lanes showed the expected band of 37,268 bp, confirming successful hybridization of the *zmm28* probe.

Hybridization of the *mo-pat* probe to *Nco* I-digested genomic DNA resulted in a single band of approximately 7,000 bp in all five generations of DP202216 maize samples analyzed (Table 6, Figure 14). This result confirmed that the 3' border fragment, containing the *mo-pat* gene in the DP202216 insertion, is intact and stable across the five generations of DP202216 maize. The plasmid lanes showed the expected band of 12,605 bp, confirming successful hybridization of the *mo-pat* probe.

The Southern blot analysis with *zmm28* and *mo-pat* gene probes showed that the 5' and 3' genomic borders of the DP202216 insertion are intact and stable across five generations of DP202216 maize during the breeding process.

Materials and methods for Southern blot analysis of DP202216 maize are described in Appendix 3.

Table 5. Description of DNA Probes used for Southern Hybridization

Probe Lot Number	Genetic Element/ Probe Name	Probe Length bp	Position on PHP40099 T-DNA (bp to bp) ¹
2018-DP-0001	<i>zmm28</i>	901	2,664 to 3,564
2018-DP-0002	<i>mo-pat</i>	660	6,135 to 6,794

¹: The probe position is based on the PHP40099 T-DNA map (Figure 4).

Table 6. Predicted and Observed Hybridization Bands on Southern Blots; *Nco* I Digest

Probe Name	Predicted and Observed Fragment Size from Plasmid PHP40099 (bp)	Predicted Fragment Size from PHP40099 T-DNA (bp)	Observed Fragment Size in DP202216 Maize ¹ (bp)	Figure
<i>zmm28</i>	37,268	>3,538	~10,000 ~12,000* ~8,500* ~6,500* ~5,500* ~4,500* ~4,200*** ~3,800** ~3,400** ~3,000* ~2,500* ~2,200*** ~1,800*** ~1,400*	13
<i>mo-pat</i>	12,605	>3,932	~7,000	14

An (*) and gray shading indicates the designated bands due to hybridization to endogenous sequences. These bands were identified in the maize control lines [] that were analyzed.

** : Endogenous bands present in DP202216 maize of [] generations and [] control maize samples.

*** : Endogenous bands present in DP202216 maize of [] generations and [] control maize samples.

¹: Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker III and VII fragments on the Southern blots. Due to inability to determine the exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.

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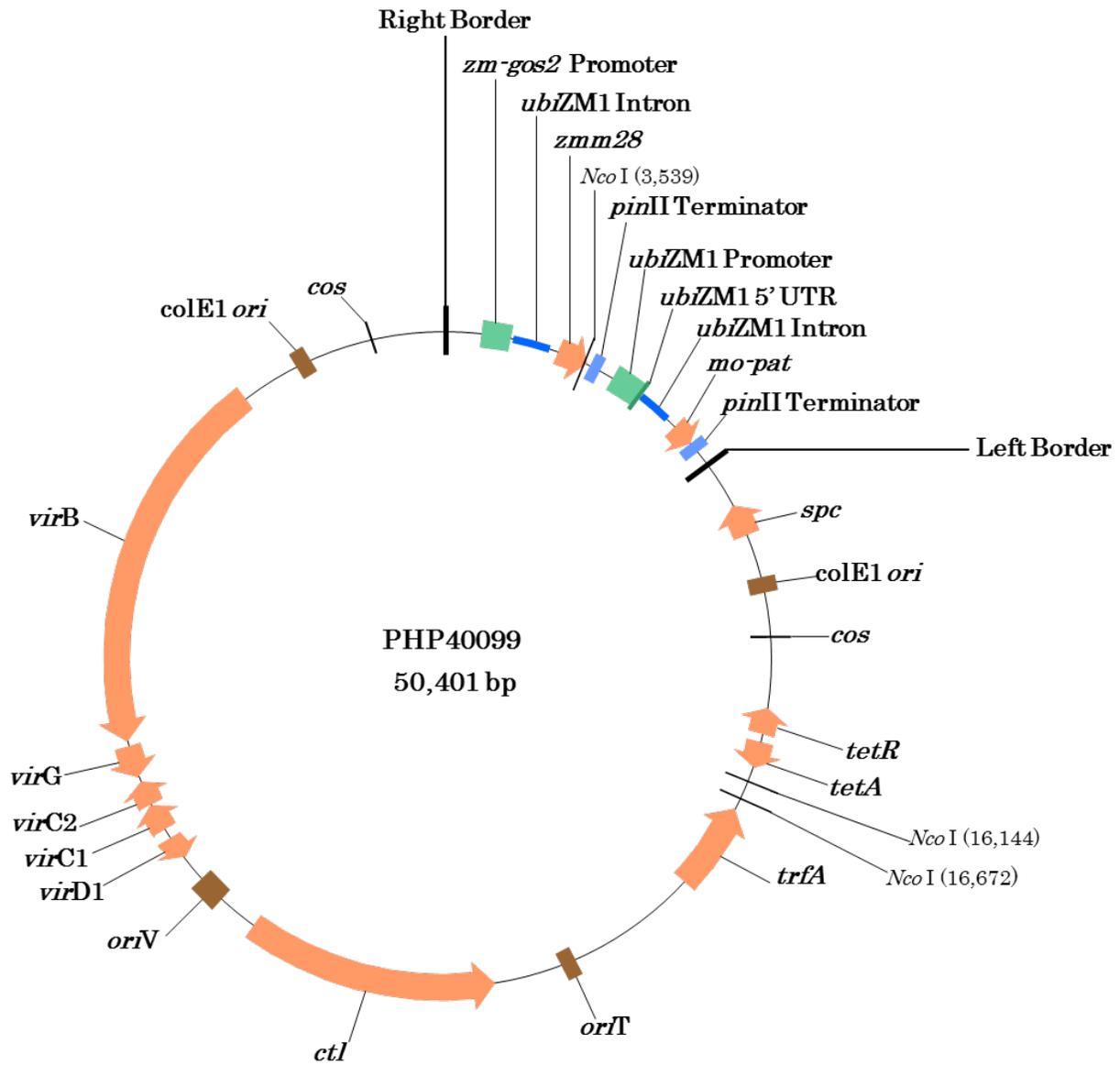
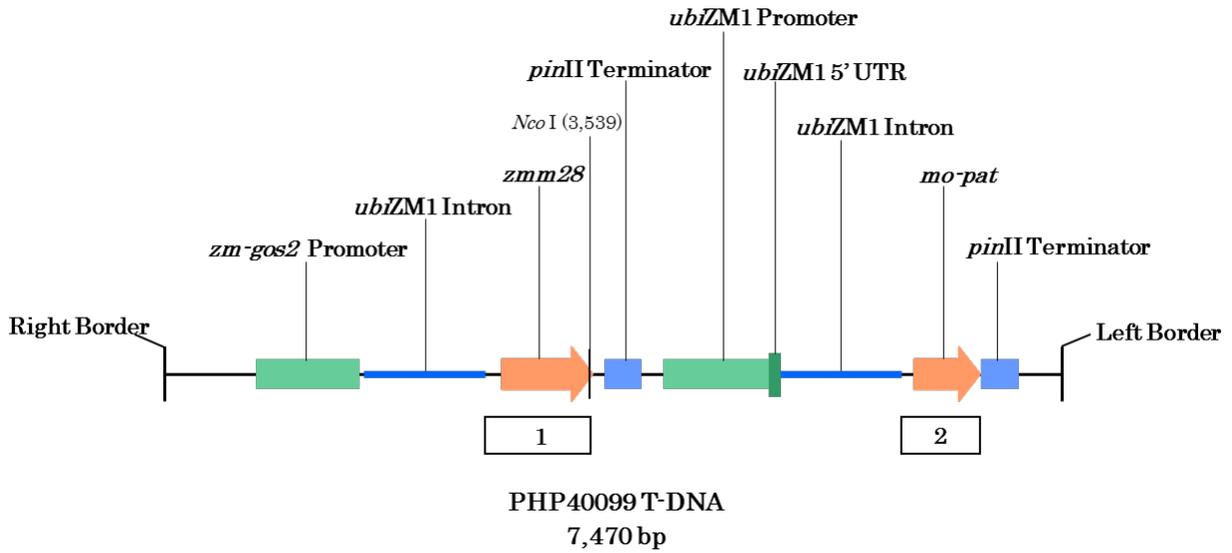


Figure 10. Map of Plasmid PHP40099 with Restriction Sites for Southern Blot Analysis

Plasmid map of PHP40099 indicating *Nco I* restriction enzyme sites with base pair positions and the *zmm28* and *mo-pat* coding and regulatory regions. The Right Border and Left Border flank the T-DNA (Figure 4) that was transferred during *Agrobacterium*-mediated transformation. Plasmid size is 50,401 bp.



Number	Probe Name
1	<i>zmm28</i>
2	<i>mo-pat</i>

Figure 11. Map of PHP40099 T-DNA with Restriction Sites and Southern Blot Probes

Map of PHP40099 T-DNA indicating the *Nco* I restriction enzyme site and the *zmm28* and *mo-pat* coding and regulatory regions. The locations of the Southern blot probes are shown by the boxes below the map.

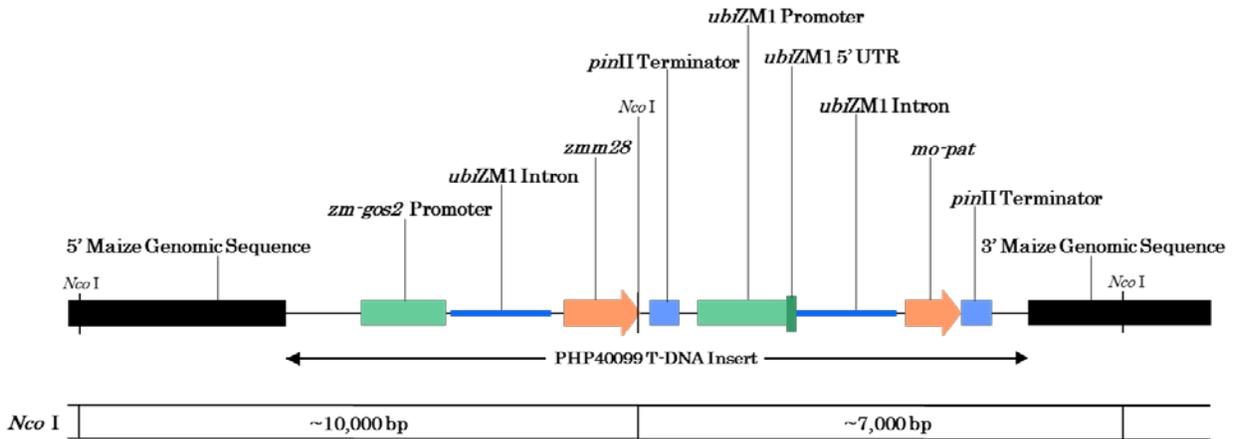
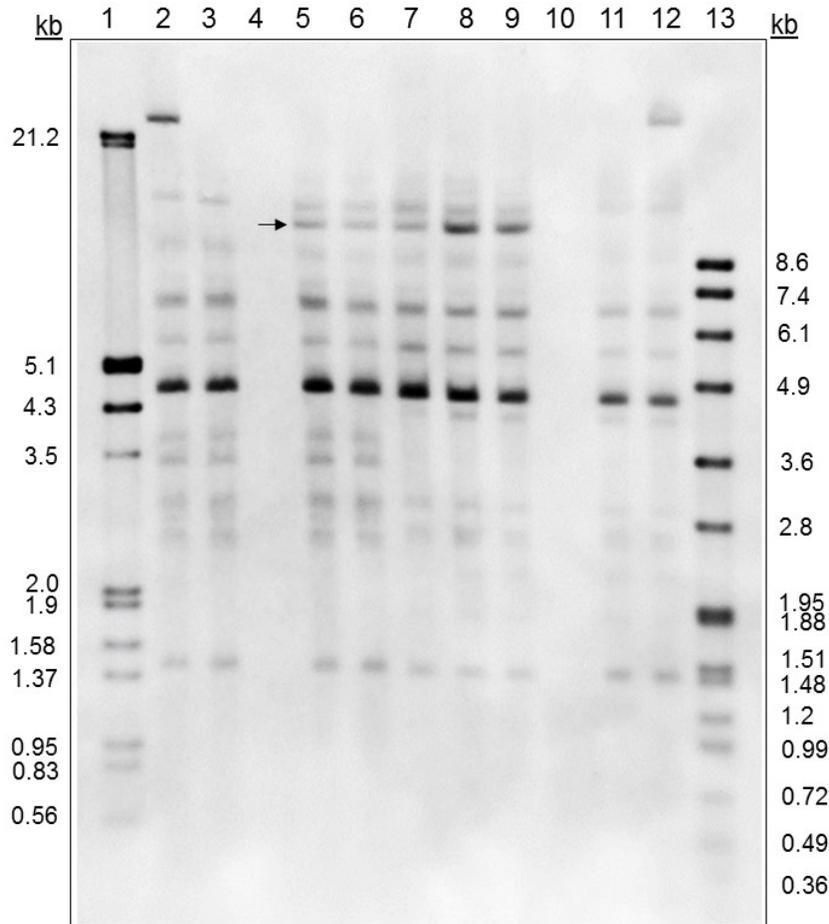


Figure 12. Map of DP202216 Maize Insertion

Map of the DP202216 maize insertion region including the *Nco* I restriction enzyme sites. The flanking maize genomic DNA is represented by the horizontal black rectangular bars. A single copy of the PHP40099 T-DNA integrated into the maize genome. *Nco* I restriction sites are indicated with the sizes of observed fragments on Southern blots shown below the map in base pairs (bp). The locations of restriction enzyme sites in the flanking maize genomic DNA are not to scale.



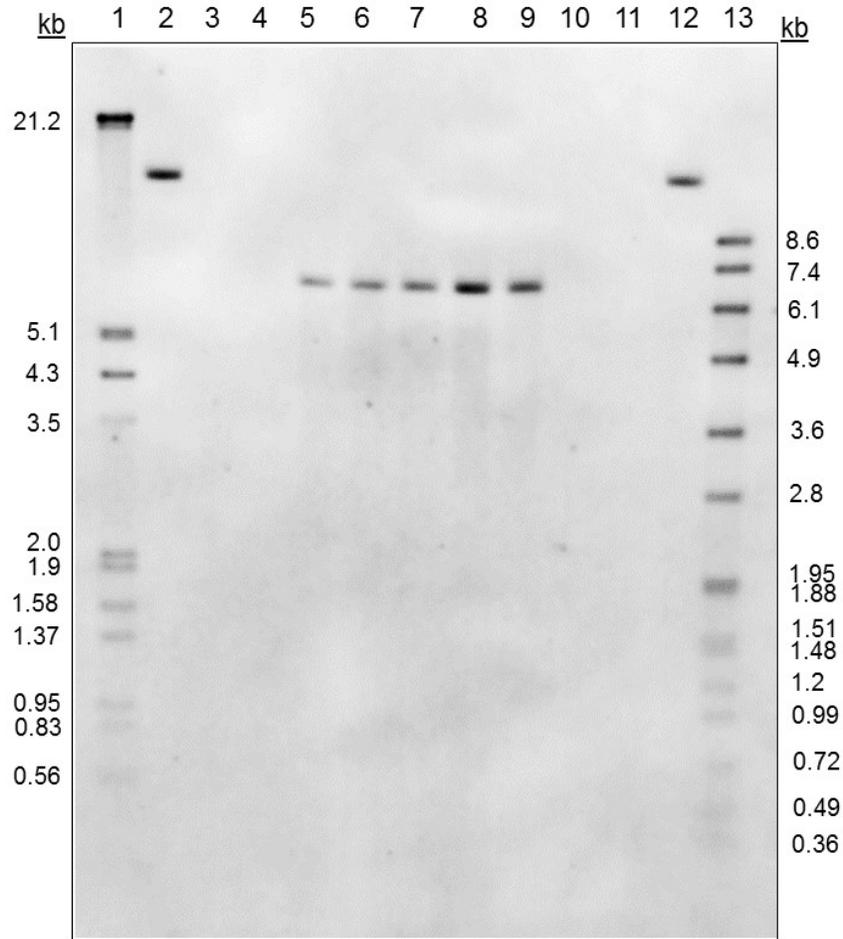
Lane	Sample	Lane	Sample
1	DIG-labeled DNA marker III	8	DP202216 maize [] generation
2	1 copy PHP40099 + [] control maize	9	DP202216 maize [] generation
3	[] control maize	10	Blank
4	Blank	11	[] control maize
5	DP202216 maize [] generation	12	1 copy PHP40099 + [] control maize
6	DP202216 maize [] generation	13	DIG-labeled DNA marker III
7	DP202216 maize [] generation		

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Figure 13. Southern Blot Analysis of DP202216 Maize; *Nco* I Digest with *zmm28* Gene Probe

Genomic DNA isolated from leaf tissues of DP202216 maize from [] generations, and [] control maize plants, were digested with *Nco* I and hybridized to the *zmm28* gene probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP40099 plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. The arrow indicates the DP202216-specific band. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).

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Lane	Sample	Lane	Sample
1	DIG-labeled DNA marker III	8	DP202216 maize [] generation
2	1 copy PHP40099 + [] control maize	9	DP202216 maize [] generation
3	[] control maize	10	Blank
4	Blank	11	[] control maize
5	DP202216 maize [] generation	12	1 copy PHP40099 + [] control maize
6	DP202216 maize [] generation	13	DIG-labeled DNA marker III
7	DP202216 maize [] generation		

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Figure 14. Southern Blot Analysis of DP202216 Maize; *Nco* I Digest with *mo-pat* Gene Probe

Genomic DNA isolated from leaf tissues of DP202216 maize from [] generations, and [] control maize plants, were digested with *Nco* I and hybridized to the *mo-pat* gene probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP40099 plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).

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V-D. Open Reading Frame Analysis

A bioinformatics assessment of translated Open Reading Frames (ORFs) of length ≥ 30 amino acids at the insertion site of DP202216 maize for similarity to known and putative allergens and toxins was conducted following established international criteria (Codex Alimentarius Commission, 2003; FAO/WHO, 2001).

None of the identified translated ORFs at the DP202216 maize insertion site returned alignments from the search against the Comprehensive Protein Allergen Resource (COMPARE) 2018 database (February 2018 available at <http://comparedatabase.org>). None of the identified translated ORFs at the DP202216 maize insertion site produced a contiguous 8-residue exact match to a sequence in the COMPARE database. These data indicate that there is no allergenicity concern regarding the identified translated ORFs at the DP202216 maize insertion site.

None of the identified translated ORFs at the DP202216 maize insertion site returned alignments from the search against the Pioneer toxin database, indicating that there is no toxicity concern regarding the identified translated ORFs at the DP202216 maize insertion site.

V-E. Inheritance and Genetic Stability of the Introduced Trait in DP202216 Maize

The inheritance of the inserted DNA during the breeding process is evaluated by examining the segregation of the genes and/or traits in multiple generations. The observed inheritance pattern predicts the segregation of these genes and/or traits as a single unit and as a single genetic locus throughout the commercial breeding process.

The inheritance pattern of the T-DNA insert within DP202216 maize was investigated by determining segregation of the *zmm28* and *mo-pat* genes within five generations []; 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 PHP40099 T-DNA insert by event-specific PCR and for the presence of each of the introduced genes by gene-specific PCR. The herbicide resistance phenotype was determined by treating plants with glufosinate herbicide and visually evaluating each plant for herbicide injury. A trait positive plant exhibited no herbicidal injury and a trait negative plant exhibited severe herbicide injury. The expected Mendelian inheritance ratio of positive and negative plants for a hemizygous trait of these populations was 3:1 for [], and 1:1 for []. All plants of the [] and [] generations of DP202216 maize were confirmed to be positive (*i.e.*, not segregating) as expected for a homozygous generation.

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Results from the segregation analysis are provided in Table 7. In every case, a positive plant tested positive for the presence of the DP202216 insertion; the *zmm28* and *mo-pat* genes; and the herbicide resistance phenotype, indicating that the inserted T-DNA and its genetic elements

within DP202216 maize 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 [], and [] generations of DP202216 maize (Table 7). A chi-square test was not performed for the [] generations as all plants were positive. 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 DP202216 maize across breeding generations. Materials and methods for the multi-generation segregation analysis are described in Appendix 4.

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Table 7. Summary of Genotypic and Phenotypic Results for Segregating Generations of DP202216 Maize

Generation	Expected Segregation Ratio	Observed Segregation ^a			Statistical Analysis	
	(Positive:Negative)	Positive	Negative	Total	Chi-Square ^b	P-Value
[]	3:1	80	20	100	1.33	0.2482
[]	1:1	54	46	100	0.64	0.4237
[]	1:1	42	58	100	2.56	0.1096
[]	Homozygous	100	0	100	--	--
[]	Homozygous	100	0	100	--	--

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^a PCR analyses (consisting of event-specific PCR analysis to confirm the presence or absence of maize event DP202216, and gene-specific PCR analysis to confirm the presence or absence of the *zmm28* and *mo-pat* genes) and herbicide (*i.e.*, glufosinate) resistance analysis were conducted for each plant in each entry. All PCR results matched the corresponding herbicide resistance result for each plant analyzed.

^b Degrees of freedom = 1.

V-F. Conclusions on Molecular Characterization and Genetic Stability of DP202216 Maize

Southern-by Sequencing and Southern blot analyses were conducted to characterize the DNA insertion in DP202216 maize. SbS analysis confirmed that a single, intact PHP40099 T-DNA was inserted into the maize genome and the integrity of the inserted DNA was maintained. Southern blot analysis on five generations 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 DP202216 maize

Bioinformatics assessment of translated open reading frames (ORFs) at the DP202216 insertion site found no similarity to known and putative allergens or toxins.

The inheritance and genetic stability of the inserted DNA was confirmed in 5 generations of DP202216 maize. The results of this analysis were consistent with the finding of a single locus of

the DP202216 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 no ORF allergen or toxin concerns in DP202216 maize.

VI. Characterization of the Proteins Introduced into DP202216 Maize

VI-A. Identity and Function of the ZMM28 and PAT Proteins Present in DP202216

Maize

DP202216 maize was genetically engineered to increase and extend the expression of the native maize ZMM28 protein relative to native ZMM28 protein expression to enhance grain yield potential. The PAT protein expressed in DP202216 confers resistance to glufosinate-ammonium herbicides and is identical to the PAT protein in products that have been previously reviewed by USDA. The identity, deduced amino acid sequence, protein mode of action, and concentrations of ZMM28 and PAT proteins in DP202216 maize tissues are described below.

VI-A.1. ZMM28 Protein

The *zmm28* gene, which encodes the ZMM28 protein, is endogenous to maize. DP202216 maize contains a *zmm28* gene cassette with a constitutive maize *zm-gos2* promoter, which increases and extends expression of the *zmm28* gene relative to the native *zmm28* gene expression. Both the introduced and native *zmm28* genes encode the ZMM28 protein. Based on *in silico* translation of the cDNA sequence in DP202216 maize, the deduced amino acid sequence of the introduced ZMM28 protein is identical to that of the native ZMM28 protein in DP202216 maize and conventional maize (represented by the B73 reference genome; Genbank accession no: NP_001105155.1). The ZMM28 protein is 251 amino acids in length and has a molecular weight of approximately 28 kDa (Figure 15).

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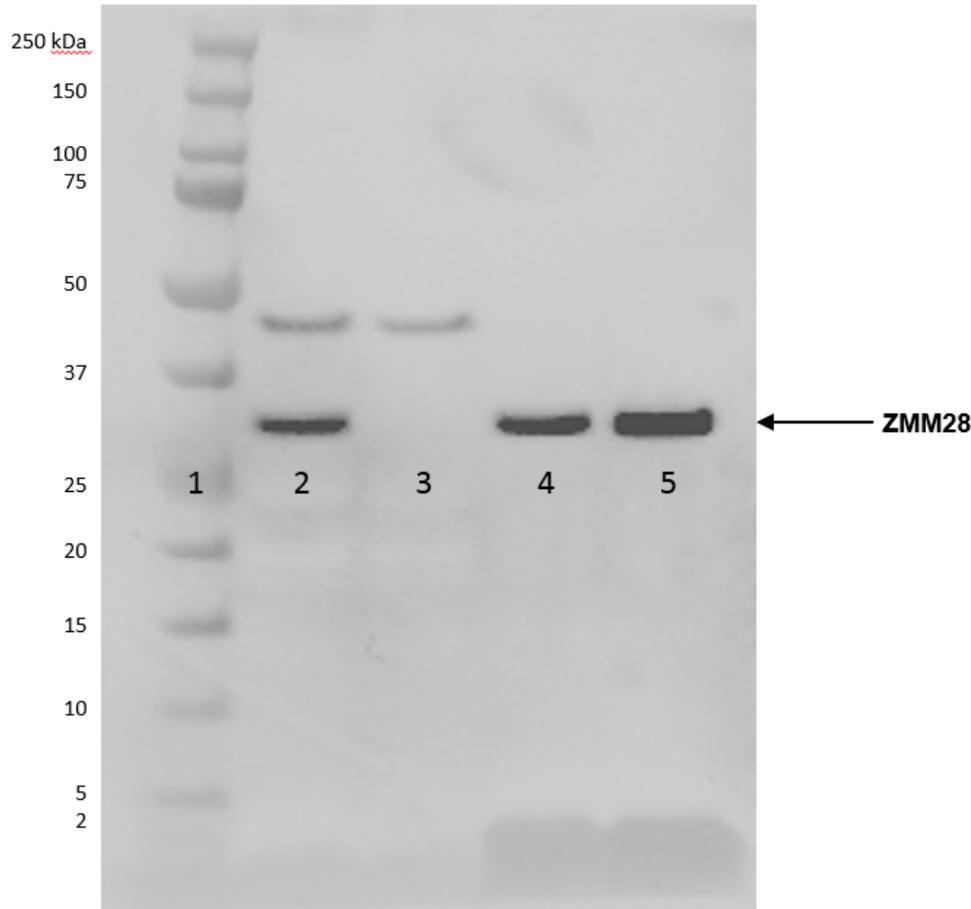
Figure 16. ZMM28 Transcription Factor Domain Structure and Protein Sequence

VI-A.1B. Equivalence of the Native and Introduced ZMM28 Protein in DP202216 Maize to the ZMM28 Protein in Near-Isoline Control Maize

Western blot analysis results (Figure 17) using a ZMM28 monoclonal antibody demonstrated expected and equivalent size (~28 kDa) of the ZMM28 protein from DP202216 maize and the ZMM28 protein from near-isoline control maize.

In the DP201226 maize R6 grain sample the ZMM28 protein was detected on a western blot (Figure 17, Lane 2) as a ~28-kDa band. In control maize R6 grain sample, a ZMM28 band was not detected on western blot, as the expression level is likely below the limit of detection (Figure 17, Lane 3). ZMM28 protein is present in both DP202216 maize and control maize V9 leaf tissues (Figure 17, Lanes 5 and 4, respectively). The relative expression level is higher in the DP202216 leaf tissue as expected, as both the endogenous and DP202216 genes are expressing simultaneously. The protein detected in the DP202216 maize R6 grain, DP202216 maize V9 leaf, and control maize V9 leaf is of equivalent size (~28 kDa).

Western blot analysis demonstrated that the ZMM28 protein in DP202216 maize and conventional maize has the expected and equivalent size (~28 kDa).



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker
2	DP202216 Maize - Grain R6
3	Near-Isoline Control Maize - Grain R6
4	Near-Isoline Control Maize - Leaf V9
5	DP202216 Maize - Leaf V9

Note: Kilodalton (kDa). Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight. A non-specific band (~45 kDa) was detected by the antibody in the DP202216 maize and control maize grain.

Figure 17. Western Blot Results for ZMM28 Protein Derived from DP202216 Maize and Near-Isoline Control Maize

VI-A.1C. Conclusion of Analysis of Amino Acid Sequence Alignment and Western Blot Analysis of the Introduced and Native ZMM28 Protein

Based on *in-silico* translation of the cDNA sequence in DP202216 maize, the deduced amino acid sequence of the introduced ZMM28 protein is identical to that of the native ZMM28 protein in conventional maize (B73). Western blot analysis confirmed that the introduced ZMM28 protein in DP202216 maize and the ZMM28 protein from control maize have the expected and equivalent size.

VI-A.1D. Safety of the ZMM28 Protein in DP202216 Maize

The source of the *zmm28* gene is maize, and the safety of maize for food and feed uses is well established (OECD, 2002).

The DNA insertion in DP202216 maize was sequenced and based on *in silico* translation of the cDNA sequence, the deduced amino acid sequence of the introduced ZMM28 protein is identical to the native ZMM28 protein in DP202216 maize and conventional maize. Western blot analysis confirmed the equivalent size and immunoreactivity of the ZMM28 protein from DP202216 maize and the ZMM28 protein from near-isoline control maize.

The amino acid sequence of the ZMM28 protein in DP202216 maize is equivalent to the amino acid sequence of the ZMM28 protein in several commonly consumed varieties of sweet corn, and shares homology with proteins in many other food crops, fruits, and vegetables ((Anderson et al., 2018-submitted). The homology of the ZMM28 protein in DP202216 maize to the ZMM28 protein in sweet corn varieties adds additional evidence to the history of safe use, as the ZMM28 protein has previously been widely and safely consumed in the human food supply.

DP202216 maize was genetically engineered to increase and extend expression of the *zmm28* gene relative to the native *zmm28* gene expression, resulting in increased and extended expression of the ZMM28 protein. However, the total amount of ZMM28 protein in DP202216 maize tissues remains low (part per billion range; refer to section VI-B below). In R6 grain, the concentration of the ZMM28 protein is comparable to the ZMM28 protein concentrations detected in the R3 harvest maturity kernels from several sweet corn varieties (Anderson et al., 2018-submitted; Seminis, 2015). The estimated acute and chronic dietary exposures to the ZMM28 protein from consumption of DP202216 maize products (conservatively assuming all maize contains DP202216) are comparable to the acute and chronic dietary exposures to the ZMM28 protein from consumption of the six sweet corn varieties assessed (Anderson et al., 2018-submitted).

The ZMM28 protein is present in harvest maturity sweet corn kernels and in vegetative tissues of conventional maize lines at levels comparable to those found in DP202216 harvest maturity kernels and vegetative tissues. The ZMM28 protein in DP202216 maize is equivalent to the maize native ZMM28 protein when comparisons are made to amino acid sequence, molecular weight, and immunoreactivity.

VI-A.2. PAT Protein

VI-A.2A. Amino Acid Sequence of the PAT Protein

The gene encoding the PAT protein in DP202216 maize, referred to as the *mo-pat* gene, was isolated from *Streptomyces viridochromogenes* with codon-optimization for expression in maize. The deduced amino acid sequence from the translation of the *mo-pat* gene is identical to the deduced amino acid sequence from the translation of the *pat* gene. The PAT protein encoded by the *pat* and *mo-pat* genes is 183 amino acids in length and has a molecular weight of approximately 21 kDa (Figure 18).

PAT(<i>pat</i>)	1	MSPERRPVEI	RPATAADMAA	VCDIVNHYIE	TSTVNFRTPEP	QTPQEWIDDL
PAT(<i>mo-pat</i>)	1	MSPERRPVEI	RPATAADMAA	VCDIVNHYIE	TSTVNFRTPEP	QTPQEWIDDL
PAT(<i>pat</i>)	51	ERLQDRYPWL	VAEVEGVVAG	IAYAGPWKAR	NAYDWTVEST	VYVSHRHQRL
PAT(<i>mo-pat</i>)	51	ERLQDRYPWL	VAEVEGVVAG	IAYAGPWKAR	NAYDWTVEST	VYVSHRHQRL
PAT(<i>pat</i>)	101	GLGSTLYTHL	LKSMEAQGFK	SVVAVIGLPN	DPSVRLHEAL	GYTARGTLRA
PAT(<i>mo-pat</i>)	101	GLGSTLYTHL	LKSMEAQGFK	SVVAVIGLPN	DPSVRLHEAL	GYTARGTLRA
PAT(<i>pat</i>)	151	AGYKHGGWHD	VGFWQRDFEL	PAPPRPVRPV	TQI*	
PAT(<i>mo-pat</i>)	151	AGYKHGGWHD	VGFWQRDFEL	PAPPRPVRPV	TQI*	

Figure 18. Sequence Alignment of the Deduced Amino Acid Sequence of the PAT Protein Encoded by *mo-pat* and *pat* Genes

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

VI-A.2B. PAT Protein Function and Activity

The *mo-pat* gene expresses the PAT protein that confers resistance to glufosinate-ammonium, the active ingredient in phosphinothricin herbicides. The PAT protein is 183 amino acids residues in length and has a molecular weight of approximately 21 kDa (Figure 18). This protein is identical to the protein found in a number of authorized events across several different crops that are currently in commercial use. Maize containing the PAT protein has been commercially grown in the United States since 1996. PAT protein safety has been reviewed and authorized for food and feed use by regulatory authorities in 20 different countries and/or regions. Authorizations for GE

plants that express the PAT protein have been issued in 7 species of plants and total over 450 authorized uses (ILSI, 2016).

The mode of action of the PAT protein has been previously characterized and described (CERA, 2011; Hérouet et al., 2005). The PAT protein confers resistance to glufosinate-ammonium, the active ingredient in phosphinothricin herbicides. Glufosinate chemically resembles the amino acid glutamate and acts to inhibit an enzyme, called glutamine synthetase, which is involved in the synthesis of glutamine. Glutamine synthetase is also involved in ammonia detoxification. Due to its similarity to glutamate, glufosinate blocks the activity of glutamine synthetase, resulting in reduced glutamine levels and a corresponding increase in concentrations of ammonia in plant tissues, leading to cell membrane disruption and cessation of photosynthesis resulting in plant death. The PAT protein confers resistance to glufosinate-ammonium herbicides by acetylating phosphinothricin, an isomer of glufosinate-ammonium, thus detoxifying the herbicide (CERA, 2011; Hérouet et al., 2005).

VI-A.2C. Equivalence of the PAT Protein in DP202216 Maize to a Reference Standard PAT Protein

Western blot analysis was conducted to confirm that the molecular weight of the PAT protein from DP202216 maize has the expected and equivalent size (~21 kDa) as the microbially derived PAT protein standard. No PAT protein was detected from the near isoline control maize plant tissue (Figure 19).

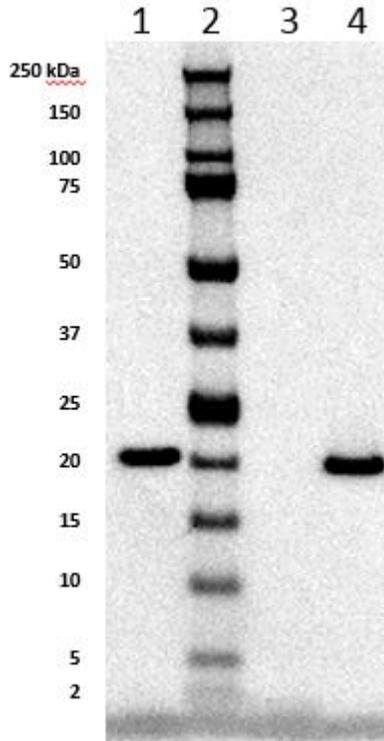


Figure 19. Western Blot Results for PAT Protein Derived from DP202216 Maize and Reference Standard Protein

Lane	Sample Identification
1	Microbially Derived PAT Protein 1.5 ng
2	Pre-stained Protein Molecular Weight Marker
3	Near-Isoline Control Maize
4	DP202216 Maize-Derived PAT Protein

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

VI-A.2D. Toxicity Assessment of the PAT Protein

DP202216 maize was evaluated by examining the toxic potential of the PAT protein. The PAT protein has been risk-assessed in previously authorized maize events, and has been determined to be unlikely to be a potential toxin to humans and animals. Previous assessments of this protein included heat lability, digestibility, and acute protein toxicity studies and are relevant for the assessment of DP202216 maize (USDA-APHIS, 2001; USDA-APHIS, 2005; USDA-APHIS, 2013). Updated bioinformatic analyses support the original conclusions that the PAT protein is unlikely to be a toxin. These data support the conclusion that the PAT protein in DP202216 maize is safe for the food and feed supply.

VI-A.2E. *In silico* Toxicity Evaluation of PAT Protein

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

One of the most important metrics of an alignment between sequences is the *E*-value. This metric represents the probability that an alignment is due to chance and can be used to evaluate the potential biological significance of the alignment. The *E*-value depends on the overall length of the aligned sequences (including inserted gaps), the number of identical and conserved residues within the alignment, and the size of the database (Baxevanis, 2005; Pearson and Lipman, 1988). When examining an alignment between two protein sequences, a very small *E*-value ($< 1 \times 10^{-5}$) is more likely to indicate a true homology, whereas a large *E*-value ($> 1 \times 10^{-4}$) is more likely to indicate a chance event lacking in biological relevance (Pearson, 2000). Consequently, if any alignment was returned between the PAT protein sequence and a Pioneer toxin database protein sequence with an *E*-value $\leq 10^{-4}$ it would be examined more closely to determine if it might imply possible toxicity of the PAT protein.

The comparison of the PAT protein sequence to the protein sequences in the Pioneer toxin database (January 16, 2018) was conducted with BLASTP using default parameters, except that low complexity filtering was turned off, the *E*-value threshold was set to 10^{-4} , and unlimited alignments were returned. Any alignment between the PAT protein and a protein in the Pioneer toxin database with an *E*-value $\leq 10^{-4}$ was examined to determine whether the alignment might imply possible toxicity of the query sequence.

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

VI-A.2F. Heat Lability of PAT Protein

The PAT protein was tested for stability at temperatures of 60, 75, and 90 °C for periods of 10, 30, and 60 minutes (Hérouet et al., 2005). The resulting proteins were analyzed by SDS-PAGE. The PAT protein remained detectable by SDS-PAGE, *i.e.*, no protein degradation, at all

temperatures and time points tested. These results corroborated the results obtained by Wehrmann et al. (1996) showing that the PAT protein was completely heat inactivated after 10 minutes at 50 °C or higher temperatures despite the fact that the protein was not degraded.

The results from the heat lability assessments support that the PAT protein is unstable at high temperatures and will be inactivated by many of the processes involved in food or animal processing (Hérouet et al., 2005). Details regarding the materials and methods used for heat lability analysis are provided in Appendix 7.

VI-A.2G. Digestibility of PAT Protein in Simulated Gastric Fluid

The PAT protein has been shown to degrade to non-detectable levels within 5 seconds after digestion in Simulated Gastric Fluid (SGF) containing pepsin (Hérouet et al., 2005; OECD, 1999).

VI-A.2H. Acute Oral Toxicity Evaluation of PAT Protein

The PAT protein was evaluated for acute oral toxicity in mice, and the dose tested was 6,000 mg of test material per kg body weight. When adjusted for purity of the test material (84% pure or 0.84 mg PAT/mg powder); (Brooks, 2000), the dose was 5,000 mg PAT protein per kg body weight. During the two-week observation period, mortality and/or clinical or behavioral signs of pathology as well as body weights were recorded. Gross necropsies were conducted at the end of the study. The results showed no mortality occurred during the study. Additionally, no adverse clinical signs were observed during the study and no adverse findings were noted at necropsy. Therefore, the acute oral LD50 for the PAT protein in mice could not be determined and is estimated to be higher than 5,000 mg PAT protein per kg body weight. Details regarding the materials and methods used for acute oral toxicity analysis are provided in Appendix 8

VI-A.2I. Allergenicity Assessment of the PAT Protein

***In silico* Allergenicity Evaluation of PAT Protein in DP202216 Maize**

Assessing expressed proteins for potential cross-reactivity with known or putative allergens is a critical part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically engineered plant products (Codex Alimentarius Commission, 2003). A bioinformatic assessment of the PAT protein for potential cross-reactivity with known or putative allergens was conducted according to relevant guidelines (Codex Alimentarius Commission, 2003; FAO/WHO, 2001).

Two separate searches for the PAT protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2018 database (February 2018) available at <http://comparedatabase.org>. This peer-reviewed database is a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergenicity Technical Committee (PATC) and is comprised of 2,038 sequences. The first search used the PAT protein sequence as the query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the allergen sequences. The

search was conducted using default parameters, except the *E*-score threshold was set to 10^{-4} . An *E*-score threshold of 10^{-4} has been shown to be an appropriate value for allergenicity searches (Mirsky et al., 2013). The generated alignments were examined to identify any that are 80 residues or longer and possess a sequence identity of 35% or greater. The second search used a Perl script developed by Pioneer (runLinearEpitopeScreen.pl) to identify any contiguous 8-residue identical matches between the PAT protein sequence and the allergen sequences.

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

VI-B. Concentration of ZMM28 and PAT Proteins in DP202216 Maize

The expression levels of ZMM28 and PAT proteins were evaluated in DP202216 maize using quantitative enzyme-linked immunosorbent assays (ELISA) or a western blot method. For analysis of ZMM28 and PAT protein concentrations, tissue samples were collected during the 2017 growing season at six sites in maize-growing regions of the United States (one site in Iowa, Indiana, Missouri, Nebraska, and Pennsylvania) and Canada (one site in Ontario). Each site included DP202216 maize and non-genetically engineered (non-GE) near-isoline control maize (referred to as control maize). Each field site was arranged into a randomized complete block design containing four blocks. Procedures employed to control the introduction of experimental bias included the use of non-systematic selection of trial and plot areas within each site, randomization of maize entries within each block, and uniform maintenance treatments across each plot area.

Plant tissue samples were collected throughout the growing season at various growth developmental stages and processed as described in Appendix 5. Time points for sampling were chosen to determine the range of protein concentrations throughout the growing season and for their relevance to typical maize production practices. The R4 stage of the whole plant sample (*i.e.*, forage) is the stage at which growers harvest plants for silage for animal feed. Grain is normally harvested at the R6 stage of development and is used for food and feed. The following tissue samples were collected: leaf (V6, V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), root (V9, R1, R4, and R6 growth stage), forage (R4 growth stage), whole plant (V9, R1, and R6 growth stages), and grain (R6 growth stage).

The concentrations of ZMM28 and PAT proteins were determined using quantitative enzyme-linked immunosorbent assays (ELISA) that have been internally validated to demonstrate method suitability. The ZMM28 ELISA could not be validated for grain due to matrix issues,

therefore, a western blot method that was developed and internally validated was used to quantify ZMM28 protein in grain. The ZMM28 protein is expressed in both the DP202216 maize and control maize samples, therefore, expression was measured in both DP202216 and control tissue samples. The gene encoding PAT protein is not present in the control maize samples, and therefore, PAT protein was not measured in control tissue samples.

The concentration results for the ZMM28 and PAT proteins are provided in Tables 8 and 9, respectively.

VI-B.1. Concentration of ZMM28 Protein in DP202216 Maize

The ranges of ZMM28 protein mean concentrations in leaf, root, and whole-plant tissues over the course of the growing season, as well as the mean concentrations in pollen and grain, are summarized in Table 8 for DP202216 maize.

Table 8. Across-Site Summary of Expressed Trait ZMM28 Protein Concentrations

Tissue	Growth Stage	DP202216 Mean (ng/mg tissue dw)	DP202216 Range (ng/mg tissue dw)	Control Mean (ng/mg tissue dw)	Control Range (ng/mg tissue dw)
Leaf	V6	0.087 ^a	<0.054 - 0.33	0.062 ^a	<0.054 - 0.28
	V9	0.28	0.066 - 0.72	0.21	0.060 - 0.56
	R1	0.32	0.084 - 0.66	0.22 ^a	<0.054 - 0.44
	R4	0.12 ^a	<0.054 - 0.22	0.079 ^a	<0.054 - 0.14
	R6	<0.054	<0.054	ND	<0.054
Pollen	R1	0.015 ^a	<0.028 - 0.028	ND	<0.028
Root	V9	0.031	<0.027 - 0.078	0.019	<0.027 - 0.051
	R1	0.015	<0.027 - 0.029	0.016	<0.027 - 0.042
	R4	0.019	<0.027 - 0.042	ND	<0.027
	R6	0.015	<0.027 - 0.042	0.014	<0.027 - 0.033
Forage	R4	0.049 ^a	<0.036 - 0.12	0.029 ^a	<0.036 - 0.058
Whole Plant	V9	0.23	0.16 - 0.36	0.20	0.11 - 0.34
	R1	0.18	0.12 - 0.26	0.14	0.080 - 0.20
	R6	0.019 ^a	<0.036 - 0.040	0.019 ^a	<0.036 - 0.044
Grain	R6	0.012 ^a	<0.0069 - 0.029	ND	<0.0069

Note: Growth stages (Abendroth et al., 2011). Lower limit of quantitation (LLOQ) = 0.054 ng/mg tissue dry weight. Not determined (ND); all samples were below the LLOQ. In DP202216 maize, the ZMM28 expression results represent a combination of both native and introduced ZMM28 protein.

^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 mean.

VI-B.2. Concentration of PAT Protein in DP202216 Maize

The ranges of PAT protein mean concentrations in leaf, root, and whole-plant tissues over the course of the growing season, as well as the mean concentrations in pollen and grain, are summarized in Table 9 for DP202216 maize.

Table 9. Across-site Summary of Expressed Trait PAT Protein Concentrations

Tissue	Growth Stage	DP202216 Mean (ng/mg tissue dw)	DP202216 Range (ng/mg tissue dw)
Leaf	V6	25	14 - 40
	V9	20	9.6 - 46
	R1	41	27 - 56
	R4	88	30 - 190
	R6	<0.11	<0.11
Pollen	R1	76	66 - 110
Root	V9	17	0.072 – 30
	R1	7.4	2.7 - 15
	R4	11	4.5 – 20
	R6	11 ^a	<0.054 - 23
Forage	R4	32	16 - 48
Whole Plant	V9	32	20 - 46
	R1	26	15 - 36
	R6	21	0.52 - 68
Grain	R6	15	7.5 - 21

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 mean and standard deviation.

VI-B.3. DP202216 Expressed Trait Protein Conclusion

Amino acid sequence and equivalency analyses confirmed the native ZMM28 protein and the introduced ZMM28 protein in DP202216 maize are equivalent. Western blot analysis confirms that the introduced ZMM28 protein in DP202216 maize and the ZMM28 protein from conventional maize have equivalent size.

DP202216 maize expresses more ZMM28 protein in tissues; however, the concentrations remain in the part per billion range. The ZMM28 protein is found in maize varieties consumed by humans and livestock and has a history of consumption.

Amino acid sequence and western blot analyses confirmed that the DP202216 maize-derived PAT protein is the same as the PAT protein present in previously authorized events. The PAT protein is unlikely to be toxic or allergenic. The DP202216 maize expresses the PAT protein in all tissues above the assay LLOQ, except for leaf (R6 stage only).

VI-C. Human and Livestock Exposure to the ZMM28 and PAT Proteins in DP202216 Maize

The intended use(s) and degree of exposure can be considered in assessing the safety of a GE crop. This consideration includes the effect(s) (if any) of the level of the food or food product in the diet, patterns of dietary consumption, and the defining characteristics of populations that consume the crop of interest (OECD, 1993). As previously described, the ZMM28 and PAT proteins have a history of safe consumption.

The levels of expression of the ZMM28 and PAT proteins were measured in edible tissues and reported in tables 8 and 9 above. These values were then used to estimate exposure to humans and livestock as reported below.

VI-C.1. Human Food Safety of the ZMM28 and PAT Proteins in DP202216 Maize

Dietary exposures to ZMM28 and PAT proteins were calculated utilizing the mean concentrations of ZMM28 and PAT proteins in DP202216 maize grain along with consumption data from Dietary Exposure Evaluation Model – Food Commodity Intake Database (DEEM™ - FCID), Version 4.02 (DEEM/FCID, 2018). This model is commonly used by the United States EPA Office of Prevention, Pesticides and Toxic Substances to estimate human dietary exposure.

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

Conservative total replacement scenarios were utilized for both acute and chronic exposures, assuming all protein in the foodstuff was derived from DP202216 maize grain. It was assumed that no degradation of proteins occurred during processing, except in the case of corn oil, corn starch, and corn syrup where protein content is considered to be zero due to processing (CRA, 2006a; CRA, 2006b; CRA, 2006c; Hefle and Taylor, 1999). Therefore, corn oil, corn starch, and corn syrup were not relevant for exposure estimates.

Mean annual (chronic) exposure was highest for the 'children ages 3-5 years' subgroup with exposures of 0.000007 and 0.008285 mg/kg body weight/day for ZMM28 and PAT proteins, respectively. The highest 95th percentile per capita daily (acute) exposure for the ZMM28 protein was in the 'children ages 1-2 years' and 'children ages 3-5 years' subgroups with exposures of 0.000027 mg/kg body weight/day; the highest 95th percentile per capita daily (acute) exposure per for the PAT protein was in the 'children ages 3-5 years' subgroup with an exposure of

0.034106 mg/kg body weight/day. The highest 95th percentile users daily (acute) exposures for ZMM28 and PAT proteins were in the 'children ages 1-2 years' subgroup with exposures of 0.000036 and 0.044548 mg/kg body weight/day, respectively.

The actual exposure to these proteins in the diet is expected to be even lower because (1) maize grain is highly blended, thus grain containing ZMM28 and PAT proteins will be mixed with other grain potentially not containing these proteins and (2) reductions in protein concentrations will occur during processing to produce maize flour and other processed commodities.

Dietary risk can be characterized by calculating the amount of grain that would have to be consumed to expose a person to the same level of protein used in an acute toxicity study in mice where no treatment-related adverse effects were observed over a 14-day period following oral gavage with the microbially-produced protein. The estimated amount of grain is then evaluated in terms of how feasible it would be for a person to eat that amount of grain in one day.

In the case of PAT protein, 20,000 kg DP202216 maize grain would have to be eaten in one day by a 60-kg adult and 3,333 kg eaten by a 10-kg child to equal the amount consumed by mice in a 14-day acute toxicology study where no treatment-related adverse effects were observed (Brooks, 2000). See Appendix 10 for more information regarding methods used for human dietary exposure.

VI-C.2. Livestock Feed Safety of the ZMM28 and PAT Proteins in DP202216 Maize

Utilizing the mean concentrations of ZMM28 and PAT proteins in grain or forage from DP202216 maize (Tables 10 and 11), daily dietary exposure (DDE) to the two proteins from consumption of DP202216 maize grain and forage were calculated for various livestock species using estimates of animal body weight, daily feed intake, and grain and forage/silage inclusion rates specific for North America (OECD, 2013; corn, field). The following conservative total replacement scenarios were utilized:

- 100% DP202216 maize grain replacement for poultry (broiler, layer, turkey), swine (breeding, finishing), cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP202216 maize forage replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP202216 maize grain and forage combination replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb)

The highest estimated DDE with 100% maize grain replacement was observed in broilers with values of 0.000818 and 1.02 mg/kg body weight/day for ZMM28 and PAT proteins, respectively. The highest estimated DDE for maize forage or maize grain and forage replacement was in dairy

cattle with DDE values from maize forage consumption of 0.00221 and 1.44 mg/kg body weight/day for ZMM28 and PAT proteins, respectively, and DDE values from maize grain + forage consumption of 0.002450 and 1.75 mg/kg body weight/day, respectively.

In practice, the actual livestock dietary exposures to these proteins are expected to be even lower than these estimates because (1) maize grain is highly blended, thus maize sources containing DP202216 proteins will be mixed with other maize grain sources potentially not containing these proteins, and (2) the estimates were highly conservative in their maize incorporation rates, not accounting for typical blending with other feedstuffs for adequate nutrient levels and least-cost formulations.

See Appendix 11 for further details regarding methods used for livestock dietary exposure.

VII. Compositional Assessment

An assessment of the compositional equivalence of a Genetically Engineered (GE) product compared to that of a non-GE comparator with a history of safe use in food and feed is a critical 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 DP202216 maize were evaluated in comparison to concurrently grown non-GE, near-isoline maize (referred to as control maize) to identify statistical differences, and subsequently were evaluated in the context of normal ranges of variation established from multiple sources of conventional maize data.

Nutrient composition analysis of DP202216 maize included proximates, fiber, minerals, fatty acids, amino acids, vitamins, secondary metabolites, and anti-nutrients. 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

Tissue samples for DP202216 maize and control maize were generated during the 2017 growing season at eight different sites in maize-growing regions of the United States (one site in Iowa, Illinois, Indiana, Missouri, Nebraska, Pennsylvania, and Texas) and Canada (one site in Ontario). A randomized complete block design with four blocks was utilized at each site. Each block included DP202216 maize, control maize, and conventional maize lines. Forage at R4 and grain at R6 growth stages were collected and analyzed for key nutritional components. All samples were collected from impartially selected, healthy, representative plants. Maize growth stages, sample collection and processing methods are provided in Appendix 9.

VII-B. Determination of Nutrient Composition Analyte Concentrations

Forage and grain samples collected during 2017 field trials were analyzed by EPL Bio Analytical Services. 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 Part 160. The analytical procedures used by EPL Bio Analytical Services were validated methods. The majority were based on methods published by the AOAC (Association of Analytical Chemists), 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 9.

VII-C. Assessment of Nutrient Composition Data

A total of 70 analytes were included in the statistical analysis of nutrient composition results, which included 69 original analytes as well as one additional calculated analyte (total tocopherols). A total of 68 analytes (9 analytes from forage and 59 analytes from grain) were analyzed using mixed model analysis. A total of 2 analytes from grain were analyzed using Fisher's exact test because the majority (*i.e.*, greater than or equal to 50%, but less than 100%) of sample values for either DP202216 maize or the control maize were below the LLOQ.

If a statistically significant difference was identified in the across-site analysis between DP202216 maize and control maize, the respective DP202216 maize data range was compared to a tolerance interval, and if one or more individual values of DP202216 maize were outside the tolerance interval, the DP202216 maize data range was compared to a literature range. If one or more individual values of DP202216 maize were outside the literature range (or a literature range was not available), the DP202216 maize data range was compared to the in-study reference range. 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.

Nutrient composition analysis results are provided in Tables 10-20. Details regarding statistical analysis methods are provided in Appendix 9.

VII-C.1. Proximates, Fiber, and Minerals in DP202216 Maize Forage

Proximates, fiber, and minerals were analyzed in forage derived from DP202216 maize and control maize. Results are shown in Table 10. No statistically significant differences (P-value < 0.05) were observed between DP202216 maize and control maize.

The results of the analysis of proximates, fiber, and minerals in maize forage demonstrate that DP202216 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 10. Proximates, Fiber, and Mineral Results for DP202216 Maize Forage

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Crude Protein	Mean	8.32	8.41			
	Range	6.30 - 11.2	6.20 - 10.8			
	Confidence Interval	7.59 - 9.05	7.68 - 9.15	4.30 - 12.6	3.14 - 16.32	5.80 - 11.8
	Adjusted P-Value	--	0.830			
	P-Value	--	0.586			
Crude Fat	Mean	3.86	4.10			
	Range	2.29 - 5.19	1.99 - 6.18			
	Confidence Interval	3.44 - 4.27	3.68 - 4.51	1.04 - 5.46	ND - 6.755	2.00 - 5.91
	Adjusted P-Value	--	0.517			
	P-Value	--	0.239			
Crude Fiber	Mean	20.0	19.8			
	Range	14.4 - 27.5	15.4 - 26.7			
	Confidence Interval	18.4 - 21.5	18.3 - 21.4	14.3 - 31.0	12.5 - 42	13.2 - 26.8
	Adjusted P-Value	--	0.942			
	P-Value	--	0.873			
ADF	Mean	25.9	25.9			
	Range	17.2 - 36.2	18.3 - 35.5			
	Confidence Interval	23.4 - 28.4	23.4 - 28.4	18.7 - 39.6	9.90 - 47.39	16.4 - 36.1
	Adjusted P-Value	--	0.993			
	P-Value	--	0.981			
NDF	Mean	40.9	41.5			
	Range	30.7 - 53.8	28.5 - 52.7			
	Confidence Interval	37.7 - 44.2	38.3 - 44.7	34.0 - 62.6	20.29 - 67.80	26.1 - 54.6
	Adjusted P-Value	--	0.875			
	P-Value	--	0.707			
Ash	Mean	4.31	4.30			
	Range	2.09 - 6.64	1.15 - 8.20			
	Confidence Interval	3.34 - 5.27	3.33 - 5.27	2.66 - 10.0	0.66 - 13.20	1.86 - 8.88
	Adjusted P-Value	--	0.993			
	P-Value	--	0.974			
Carbohydrates	Mean	83.6	83.1			
	Range	79.3 - 88.5	77.9 - 87.7			
	Confidence Interval	81.9 - 85.3	81.4 - 84.7	76.5 - 89.5	73.3 - 92.9	77.4 - 88.9
	Adjusted P-Value	--	0.459			
	P-Value	--	0.184			
Calcium	Mean	0.210	0.216			
	Range	0.0777 - 0.315	0.157 - 0.398			
	Confidence Interval	0.178 - 0.243	0.184 - 0.249	0.0931 - 0.537	0.06 - 0.58	0.119 - 0.400
	Adjusted P-Value	--	0.815			
	P-Value	--	0.503			
Phosphorus	Mean	0.253	0.257			
	Range	0.149 - 0.349	0.125 - 0.347			
	Confidence Interval	0.216 - 0.291	0.220 - 0.295	0.0956 - 0.454	0.07 - 0.55	0.109 - 0.344
	Adjusted P-Value	--	0.830			
	P-Value	--	0.582			

Note: Proximates, fiber, and minerals unit of measure is % dry weight. 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. ADF (Acid Detergent Fiber), NDF (Neutral Detergent Fiber)

VII-C.2. Proximates and Fiber in DP202216 Maize Grain

Proximates and fiber were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in Table 11. No statistically significant differences (P-value < 0.05) were observed between DP202216 maize and control maize.

The results of the analysis of proximates and fiber in maize grain demonstrate that DP202216 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 11. Proximates and Fiber Results for DP202216 and Control Maize Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Total Dietary Fiber	Mean	8.88	8.94			
	Range	6.81 - 12.7	6.96 - 13.2			
	Confidence Interval	8.10 - 9.67	8.16 - 9.73	5.91 - 15.8	6.68 - 35.31	6.53 - 15.2
	Adjusted P-Value	--	0.942			
	P-Value	--	0.879			
Crude Protein	Mean	8.36	8.58			
	Range	7.08 - 10.5	7.02 - 10.6			
	Confidence Interval	7.78 - 8.93	8.01 - 9.16	7.18 - 13.2	5.72 - 17.26	7.12 - 11.7
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0670			
Crude Fat	Mean	4.19	4.21			
	Range	3.09 - 5.36	3.10 - 5.35			
	Confidence Interval	3.93 - 4.46	3.95 - 4.48	2.58 - 6.00	1.363 - 7.830	2.45 - 5.86
	Adjusted P-Value	--	0.942			
	P-Value	--	0.887			
Crude Fiber	Mean	2.36	2.39			
	Range	1.71 - 3.14	1.13 - 3.06			
	Confidence Interval	2.19 - 2.52	2.23 - 2.55	1.44 - 3.48	0.49 - 5.5	1.18 - 4.04
	Adjusted P-Value	--	0.849			
	P-Value	--	0.649			
ADF	Mean	4.24	4.55			
	Range	3.45 - 5.77	2.87 - 6.88			
	Confidence Interval	3.97 - 4.52	4.27 - 4.82	2.64 - 6.26	1.41 - 11.34	2.89 - 7.94
	Adjusted P-Value	--	0.459			
	P-Value	--	0.118			
NDF	Mean	9.74	9.48			
	Range	6.88 - 11.4	6.86 - 11.3			
	Confidence Interval	9.26 - 10.2	9.00 - 9.96	7.22 - 20.8	4.28 - 22.64	5.87 - 12.7
	Adjusted P-Value	--	0.545			
	P-Value	--	0.273			
Ash	Mean	1.27	1.30			
	Range	0.810 - 1.43	0.952 - 1.54			
	Confidence Interval	1.15 - 1.39	1.17 - 1.42	0.976 - 1.80	0.616 - 6.282	0.830 - 1.63
	Adjusted P-Value	--	0.459			
	P-Value	--	0.112			
Carbohydrates	Mean	86.1	85.9			
	Range	83.6 - 88.0	83.9 - 88.5			
	Confidence Interval	85.4 - 86.9	85.1 - 86.6	80.2 - 88.0	77.4 - 89.7	81.5 - 88.1
	Adjusted P-Value	--	0.459			
	P-Value	--	0.130			

VII-C.3. Fatty Acids in DP202216 Maize Grain

Fatty acids were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in Tables 12 and 13. No statistically significant differences (P-value < 0.05) were observed between DP202216 maize and control maize.

The results of the analysis of fatty acids in maize grain demonstrate that DP202216 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 12. Fatty Acids Results for DP202216 Maize Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Lauric Acid (C12:0)	Mean	<LLOQ ^a	<LLOQ ^a			
	Range	<LLOQ ^a	<LLOQ ^a			
	Confidence Interval	NA	NA	0.00 - 0.209 ^b	ND - 0.698	<LLOQ ^a
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Myristic Acid (C14:0)	Mean	<LLOQ ^a	<LLOQ ^a			
	Range	<LLOQ ^a	<LLOQ ^a			
	Confidence Interval	NA	NA	0.00 - 0.267 ^b	ND - 0.288	<LLOQ ^a
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Palmitic Acid (C16:0)	Mean	10.6	10.6			
	Range	10.3 - 11.7	10.3 - 11.3			
	Confidence Interval	10.4 - 10.9	10.4 - 10.8	9.23 - 26.0	6.81 - 39.0	10.0 - 14.2
	Adjusted P-Value	--	0.867			
	P-Value	--	0.688			
Palmitoleic Acid (C16:1)	Mean	0.0775	0.0787			
	Range	0.0369 - 0.105	0.0385 - 0.107			
	Confidence Interval	0.0643 - 0.0906	0.0655 - 0.0919	0 - 0.463	ND - 0.67	0.0349 - 0.136
	Adjusted P-Value	--	0.836			
	P-Value	--	0.627			
Heptadecanoic Acid (C17:0)	Mean	<LLOQ ^a	<LLOQ ^a			
	Range	<LLOQ ^a	<LLOQ ^a			
	Confidence Interval	NA	NA	0 - 0.245	ND - 0.203	<LLOQ ^a
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Heptadecenoic Acid (C17:1)	Mean	<LLOQ ^a	<LLOQ ^a			
	Range	<LLOQ ^a	<LLOQ ^a			
	Confidence Interval	NA	NA	0.00 - 0.135 ^b	ND - 0.131	<LLOQ ^a
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Stearic Acid (C18:0)	Mean	2.06	2.09			
	Range	1.77 - 2.40	1.66 - 2.42			
	Confidence Interval	1.91 - 2.22	1.94 - 2.24	1.31 - 3.94	ND - 4.9	1.39 - 2.54
	Adjusted P-Value	--	0.545			
	P-Value	--	0.265			
Oleic Acid (C18:1)	Mean	29.9	29.9			
	Range	28.3 - 32.3	27.5 - 32.5			
	Confidence Interval	28.8 - 30.9	28.9 - 30.9	18.9 - 39.4	16.38 - 42.81	22.4 - 34.3
	Adjusted P-Value	--	0.878			
	P-Value	--	0.765			
Linoleic Acid (C18:2)	Mean	55.0	54.9			
	Range	51.3 - 56.7	51.2 - 57.3			
	Confidence Interval	53.6 - 56.4	53.5 - 56.3	28.9 - 64.4	13.1 - 67.68	45.5 - 60.6
	Adjusted P-Value	--	0.830			
	P-Value	--	0.571			
Alpha-Linolenic Acid (C18:3)	Mean	1.33	1.33			
	Range	1.20 - 1.53	1.16 - 1.56			
	Confidence Interval	1.26 - 1.40	1.26 - 1.40	0.0362 - 2.15	ND - 2.33	0.922 - 2.21
	Adjusted P-Value	--	0.944			
	P-Value	--	0.902			
Arachidic Acid (C20:0)	Mean	0.388	0.390			
	Range	0.337 - 0.498	0.344 - 0.526			
	Confidence Interval	0.353 - 0.424	0.354 - 0.426	0.296 - 0.916	0.267 - 1.2	0.296 - 0.558
	Adjusted P-Value	--	0.830			
	P-Value	--	0.576			

Table 12. Fatty Acids in DP202216 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Eicosenoic Acid (C20:1)	Mean	0.256	0.258			
	Range	0.234 - 0.290	0.236 - 0.304			
	Confidence Interval	0.243 - 0.270	0.245 - 0.271	0.0380 - 0.693	ND - 1.952	0.224 - 0.521
	Adjusted P-Value	--	0.799			
	P-Value	--	0.470			
Eicosadienoic Acid (C20:2)	Mean	<LLOQ ^a	<LLOQ ^a			
	Range	<LLOQ ^a	<LLOQ ^a			
	Confidence Interval	NA	NA	0.00 - 0.825 ^b	ND - 2.551	<LLOQ ^a
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Behenic Acid (C22:0)	Mean	0.0873	0.0871			
	Range	0.0700 - 0.182	0.0710 - 0.204			
	Confidence Interval	NA	NA	0 - 0.453	ND - 0.5	0.0691 - 0.314
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Lignoceric Acid (C24:0)	Mean	0.165	0.167			
	Range	0.0708 - 0.258	0.0712 - 0.283			
	Confidence Interval	0.0729 - 0.204	0.0823 - 0.206	0 - 0.639	ND - 0.91	0.0796 - 0.391
	Adjusted P-Value	--	0.878			
	P-Value	--	0.788			

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

^a < LLOQ, all fatty acid sample values in the current study were below the assay LLOQ. Statistical analysis was not performed for those analytes.

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

Table 13. Number of Fatty Acid Sample Values Below the Lower Limit of Quantification for DP202216 Maize Grain

Analyte	Number of Samples Below the LLOQ		Fisher's Exact Test P-Value
	Control Maize (n=32)	DP202216 Maize (n=32)	
Lauric Acid (C12:0)	32	32	--
Myristic Acid (C14:0)	32	32	--
Palmitoleic Acid (C16:1) ^a	9	8	--
Heptadecanoic Acid (C17:0)	32	32	--
Heptadecenoic Acid (C17:1)	32	32	--
Eicosadienoic Acid (C20:2)	32	32	--
Behenic Acid (C22:0)	30	30	1.00
Lignoceric Acid (C24:0) ^a	15	13	--

Note: Fatty acids unit of measure is % total fatty acids. Fatty acids analyte erucic acid (C22:1) was not statistically analyzed because all sample values in the current study and in historical conventional (non-GE) reference lines were below the lower limit of quantification (LLOQ). This analyte was excluded from the report table.

^a This analyte had <50% below-LLOQ sample values in DP202216 maize and the control maize, and was subjected to the mixed model analyses.

VII-C.4. Amino Acids in DP202216 Maize Grain

Amino acids were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in Table 14.

A statistically significant difference (P-value < 0.05) was observed between DP202216 maize and control maize mean values for glycine, methionine, and serine; however, all the individual values were within the tolerance interval, indicating DP202216 maize is within the range of normal variation for these amino acids and the statistical differences are not biologically meaningful. The non-significant FDR-adjusted P-values indicate that these differences were likely false positives.

The results of the analysis of amino acids in maize grain demonstrate that DP202216 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 14. Amino Acid Results for DP202216 Maize Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Alanine	Mean	0.609	0.623			
	Range	0.503 - 0.803	0.479 - 0.800			
	Confidence Interval	0.554 - 0.663	0.569 - 0.677	0.492 - 1.08	0.44 - 1.48	0.500 - 0.937
	Adjusted P-Value	--	0.459			
	P-Value	--	0.103			
Arginine	Mean	0.380	0.390			
	Range	0.309 - 0.429	0.315 - 0.450			
	Confidence Interval	0.356 - 0.405	0.365 - 0.414	0.317 - 0.568	0.12 - 0.71	0.305 - 0.502
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0825			
Aspartic Acid	Mean	0.530	0.540			
	Range	0.434 - 0.649	0.412 - 0.651			
	Confidence Interval	0.488 - 0.572	0.498 - 0.582	0.445 - 0.916	0.33 - 1.21	0.429 - 0.779
	Adjusted P-Value	--	0.517			
	P-Value	--	0.243			
Cystine	Mean	0.191	0.201			
	Range	0.124 - 0.228	0.126 - 0.239			
	Confidence Interval	0.177 - 0.204	0.188 - 0.214	0.132 - 0.303	0.12 - 0.51	0.0948 - 0.272
	Adjusted P-Value	--	0.468			
	P-Value	--	0.206			
Glutamic Acid	Mean	1.53	1.57			
	Range	1.23 - 2.03	1.20 - 2.03			
	Confidence Interval	1.38 - 1.68	1.42 - 1.71	1.04 - 2.70	0.97 - 3.54	1.24 - 2.38
	Adjusted P-Value	--	0.459			
	P-Value	--	0.127			
Glycine	Mean	0.350	0.362			
	Range	0.304 - 0.392	0.303 - 0.461			
	Confidence Interval	0.332 - 0.367	0.344 - 0.379	0.292 - 0.487	0.184 - 0.685	0.291 - 0.446
	Adjusted P-Value	--	0.215			
	P-Value	--	0.00731 ^a			
Histidine	Mean	0.249	0.256			
	Range	0.206 - 0.300	0.207 - 0.297			
	Confidence Interval	0.231 - 0.267	0.238 - 0.274	0.177 - 0.359	0.14 - 0.46	0.200 - 0.345
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0819			
Isoleucine	Mean	0.282	0.289			
	Range	0.231 - 0.389	0.223 - 0.386			
	Confidence Interval	0.256 - 0.308	0.263 - 0.315	0.229 - 0.494	0.18 - 0.69	0.237 - 0.421
	Adjusted P-Value	--	0.459			
	P-Value	--	0.173			
Leucine	Mean	1.01	1.03			
	Range	0.802 - 1.46	0.778 - 1.45			
	Confidence Interval	0.898 - 1.12	0.920 - 1.15	0.763 - 1.85	0.64 - 2.49	0.843 - 1.62
	Adjusted P-Value	--	0.459			
	P-Value	--	0.168			
Lysine	Mean	0.263	0.272			
	Range	0.198 - 0.319	0.220 - 0.327			
	Confidence Interval	0.246 - 0.279	0.256 - 0.288	0.186 - 0.412	0.129 - 0.668	0.127 - 0.391
	Adjusted P-Value	--	0.459			
	P-Value	--	0.146			
Methionine	Mean	0.187	0.201			
	Range	0.135 - 0.231	0.143 - 0.234			
	Confidence Interval	0.174 - 0.200	0.188 - 0.214	0.108 - 0.342	0.10 - 0.47	0.104 - 0.246
	Adjusted P-Value	--	0.334			
	P-Value	--	0.0246 ^a			

Table 14. Amino Acid Results for DP202216 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Phenylalanine	Mean	0.418	0.430			
	Range	0.293 - 0.570	0.314 - 0.567			
	Confidence Interval	0.371 - 0.465	0.383 - 0.477	0.342 - 0.736	0.24 - 0.93	0.321 - 0.626
	Adjusted P-Value	--	0.459			
	P-Value	--	0.189			
Proline	Mean	0.780	0.798			
	Range	0.649 - 1.01	0.616 - 1.01			
	Confidence Interval	0.709 - 0.851	0.727 - 0.869	0.597 - 1.25	0.46 - 1.75	0.631 - 1.11
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0912			
Serine	Mean	0.430	0.446			
	Range	0.342 - 0.526	0.346 - 0.609			
	Confidence Interval	0.395 - 0.465	0.412 - 0.481	0.296 - 0.677	0.18 - 0.91	0.356 - 0.595
	Adjusted P-Value	--	0.334			
	P-Value	--	0.0197 ^a			
Threonine	Mean	0.310	0.318			
	Range	0.265 - 0.371	0.260 - 0.374			
	Confidence Interval	0.290 - 0.330	0.298 - 0.338	0.179 - 0.476	0.22 - 0.67	0.265 - 0.413
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0519			
Tryptophan	Mean	0.0584	0.0590			
	Range	0.0358 - 0.0690	0.0366 - 0.0702			
	Confidence Interval	0.0545 - 0.0618	0.0553 - 0.0624	0.0405 - 0.0913	0.027 - 0.215	0.0356 - 0.0813
	Adjusted P-Value	--	0.867			
	P-Value	--	0.678			
Tyrosine	Mean	0.216	0.221			
	Range	0.162 - 0.283	0.157 - 0.273			
	Confidence Interval	0.197 - 0.234	0.203 - 0.239	0.164 - 0.421	0.10 - 0.73	0.176 - 0.315
	Adjusted P-Value	--	0.663			
	P-Value	--	0.341			
Valine	Mean	0.384	0.394			
	Range	0.329 - 0.485	0.316 - 0.489			
	Confidence Interval	0.357 - 0.412	0.366 - 0.421	0.318 - 0.626	0.21 - 0.86	0.325 - 0.541
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0800			

Note: Amino acids unit of measure is % dry weight.

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

VII-C.5. Minerals in DP202216 Maize Grain

Minerals were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in Tables 15 and 16. No statistically significant differences (P-value < 0.05) were observed between DP202216 maize and control maize.

The results of the analysis of minerals in maize grain demonstrate that DP202216 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 15. Minerals Results for DP202216 Maize Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Calcium	Mean	0.00342	0.00340			
	Range	0.00285 - 0.00435	0.00271 - 0.00408			
	Confidence Interval	0.00321 - 0.00364	0.00318 - 0.00361	0.00131 - 0.00784	ND - 0.101	0.00212 - 0.00595
	Adjusted P-Value	--	0.875			
	P-Value	--	0.720			
Copper	Mean	0.000128	0.000125			
	Range	<0.0000625 ^a - 0.000238	<0.0000625 ^a - 0.000212	<0.0000625 - 0.000617	ND - 0.0021	<0.0000625 ^a - 0.000169
	Confidence Interval	0.0000988 - 0.000157	0.0000955 - 0.000154			
	Adjusted P-Value	--	0.836			
	P-Value	--	0.624			
Iron	Mean	0.00168	0.00173			
	Range	0.00151 - 0.00195	0.00146 - 0.00220			
	Confidence Interval	0.00160 - 0.00177	0.00164 - 0.00181	0.00118 - 0.00261	0.0000712 - 0.0191	0.00120 - 0.00218
	Adjusted P-Value	--	0.459			
	P-Value	--	0.168			
Magnesium	Mean	0.108	0.110			
	Range	0.0876 - 0.137	0.0904 - 0.136			
	Confidence Interval	0.0993 - 0.116	0.102 - 0.119	0.0787 - 0.163	0.0035 - 1.000	0.0820 - 0.147
	Adjusted P-Value	--	0.459			
	P-Value	--	0.188			
Manganese	Mean	0.000556	0.000571			
	Range	0.000346 - 0.000801	0.000273 - 0.000850			
	Confidence Interval	0.000426 - 0.000685	0.000442 - 0.000701	0.000328 - 0.00131	0.0000312 - 0.0054	0.000289 - 0.000992
	Adjusted P-Value	--	0.468			
	P-Value	--	0.204			
Phosphorus	Mean	0.296	0.298			
	Range	0.209 - 0.367	0.205 - 0.351			
	Confidence Interval	0.262 - 0.330	0.264 - 0.332	0.204 - 0.429	0.010 - 0.750	0.189 - 0.410
	Adjusted P-Value	--	0.878			
	P-Value	--	0.775			
Potassium	Mean	0.399	0.395			
	Range	0.306 - 0.459	0.316 - 0.451			
	Confidence Interval	0.371 - 0.427	0.367 - 0.423	0.222 - 0.541	0.18 - 0.720	0.276 - 0.511
	Adjusted P-Value	--	0.836			
	P-Value	--	0.615			
Sodium	Mean	0.000158	0.000101			
	Range	<0.0000625 ^a - 0.000961	<0.0000625 ^a - 0.000726	0.00000298 - 0.00366	ND - 0.150	<0.0000625 ^a - 0.00207
	Confidence Interval	0.000102 - 0.000244	0.0000655 - 0.000156			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0926			
Zinc	Mean	0.00226	0.00226			
	Range	0.00183 - 0.00277	0.00166 - 0.00282			
	Confidence Interval	0.00205 - 0.00248	0.00205 - 0.00248	0.00140 - 0.00365	0.0000283 - 0.0043	0.00150 - 0.00295
	Adjusted P-Value	--	0.993			
	P-Value	--	0.993			

Note: Minerals unit of measure is % dry weight. Not detected (ND): one or more assay values in the published literature references were below the LLOQ and were not quantified.

^a < 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.

Table 16. Number of Minerals Sample Values Below the Lower Limit of Quantification for DP202216 Maize Grain

Analyte	Number of Samples Below the LLOQ	
	Control Maize (n=32)	DP202216 Maize (n=32)
Copper ^a	3	3
Sodium ^a	7	12

Note: Minerals unit of measure is % dry weight.

^a This analyte had <50% below-LLOQ sample values in DP202216 maize and the control maize, and was subjected to the mixed model analyses.

VII-C.6. Vitamins in DP202216 Maize Grain

Vitamins were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in Tables 17 and 18.

A statistically significant difference (P -value < 0.05) was observed between DP202216 maize and control maize mean values for vitamin B1 (thiamine) and vitamin B3 (niacin); however, all the individual values were within the tolerance interval, indicating DP202216 maize is within the range of normal variation for these vitamins and the statistical differences are not biologically meaningful. The non-significant FDR-adjusted P -values indicate that these differences were likely false positives.

The results of the analysis of vitamins in maize grain demonstrate that DP202216 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 17. Vitamins Results for DP202216 Maize Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
β-Carotene	Mean	0.983	0.962			
	Range	0.429 - 2.08	0.413 - 2.30			
	Confidence Interval	0.615 - 1.35	0.593 - 1.33	<0.0500 - 2.06 ^a	0.3 - 5.4	0.249 - 3.51
	Adjusted P-Value	--	0.815			
	P-Value	--	0.503			
Vitamin B1 (Thiamine)	Mean	2.38	2.54			
	Range	2.08 - 3.08	1.99 - 3.23			
	Confidence Interval	2.25 - 2.51	2.41 - 2.68	1.71 - 5.38	ND - 40.00	1.97 - 3.11
	Adjusted P-Value	--	0.215			
	P-Value	--	0.00466 ^b			
Vitamin B2 (Riboflavin)	Mean	<0.900 ^c	<0.900 ^c			
	Range	<0.900 ^c	<0.900 ^c			
	Confidence Interval	NA	NA	<0.900 - 2.27 ^a	ND - 7.35	<0.900 ^c
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Vitamin B3 (Niacin)	Mean	14.7	13.5			
	Range	10.9 - 22.7	9.33 - 16.2			
	Confidence Interval	13.9 - 15.6	12.7 - 14.4	7.86 - 25.2	ND - 70	9.49 - 66.0
	Adjusted P-Value	--	0.215			
	P-Value	--	0.00947 ^b			
Vitamin B5 (Pantothenic Acid)	Mean	5.11	4.71			
	Range	3.62 - 7.10	3.16 - 6.22			
	Confidence Interval	4.66 - 5.57	4.25 - 5.17	3.05 - 7.66	3.0 - 14	3.08 - 6.51
	Adjusted P-Value	--	0.459			
	P-Value	--	0.152			
Vitamin B6 (Pyridoxine)	Mean	4.54	4.44			
	Range	2.81 - 9.48	2.23 - 8.15			
	Confidence Interval	3.95 - 5.22	3.87 - 5.11	1.37 - 8.67	ND - 12.14	2.51 - 10.7
	Adjusted P-Value	--	0.878			
	P-Value	--	0.761			
Vitamin B9 (Folic Acid)	Mean	0.923	0.854			
	Range	0.565 - 2.50	0.235 - 1.72			
	Confidence Interval	0.795 - 1.07	0.735 - 0.992	0.319 - 2.41	ND - 3.50	0.461 - 2.70
	Adjusted P-Value	--	0.794			
	P-Value	--	0.456			
α-Tocopherol	Mean	4.28	4.44			
	Range	0.969 - 7.63	1.07 - 8.92			
	Confidence Interval	3.08 - 5.48	3.24 - 5.64	0 - 25.1	ND - 68.67	<0.500 ^c - 21.3
	Adjusted P-Value	--	0.830			
	P-Value	--	0.574			
β-Tocopherol	Mean	<0.500 ^c	<0.500 ^c			
	Range	<0.500 ^c	<0.500 ^c			
	Confidence Interval	NA	NA	<0.500 - 1.10 ^a	ND - 19.80	<0.500 ^c
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
γ-Tocopherol	Mean	25.9	26.9			
	Range	10.8 - 35.6	11.4 - 36.3			
	Confidence Interval	21.9 - 30.0	22.8 - 30.9	0 - 46.5	ND - 58.61	3.06 - 42.7
	Adjusted P-Value	--	0.740			
	P-Value	--	0.392			

Table 17. Vitamins Results for DP202216 Maize in Grain (continued)

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
δ-Tocopherol	Mean	0.519	0.533			
	Range	<0.500 ^c - 1.16	<0.500 ^c - 1.13			
	Confidence Interval	NA	NA	<0.500 - 2.61 ^a	ND - 14.61	<0.500 ^c - 1.14
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Total Tocopherols	Mean	31.0	32.1			
	Range	12.3 - 42.2	13.6 - 42.8			
	Confidence Interval	26.7 - 35.3	27.8 - 36.4	0 - 61.0	ND - 89.91	5.33 - 52.1
	Adjusted P-Value	--	0.788			
	P-Value	--	0.438			

Note: Vitamins unit of measure is mg/kg dry weight. Not detected (ND): one or more assay values in the published literature references were below the LLOQ and were not quantified. Not applicable (NA): mixed model analysis was not performed or confidence interval was not determined.

^a An historical reference data range was provided as tolerance interval was not calculated since the data did not meet the assumptions of any tolerance interval calculation method.

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

^c < LLOQ (where a numerical number for LLOQ value is reported, e.g. <0.900 for vitamin B2), one or more vitamin sample values were below the assay LLOQ.

Table 18. Number of Vitamins Sample Values Below the Lower Limit of Quantification for DP202216 Maize Grain

Analyte	Number of Samples Below the LLOQ		Fisher's Exact Test P-Value
	Control Maize (n=32)	DP202216 Maize (n=32)	
Vitamin B2 (Riboflavin)	32	32	--
β-Tocopherol	32	32	--
δ-Tocopherol	18	18	1.00

Note: Vitamins unit of measure is mg/kg dry weight.

VII-C.7. Secondary Metabolites and Anti-Nutrients in DP202216 Maize Grain

Secondary metabolites and anti-nutrients were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in Tables 19 and 20. No statistically significant differences (P-value < 0.05) were observed between DP202216 maize and control maize.

The results of the analysis of secondary metabolites and anti-nutrients in maize grain demonstrate that DP202216 maize is comparable to conventional maize represented by non-GE near-isoline control maize and non-GE conventional maize.

Table 19. Secondary Metabolites and Anti-Nutrients Results for DP202216 Maize Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
<i>p</i> -Coumaric Acid	Mean	0.0233	0.0242			
	Range	0.0182 - 0.0296	0.0200 - 0.0297			
	Confidence Interval	0.0212 - 0.0254	0.0221 - 0.0264	0.00715 - 0.0521	ND - 0.08	0.0150 - 0.0505
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0518			
Ferulic Acid	Mean	0.207	0.213			
	Range	0.170 - 0.249	0.190 - 0.254			
	Confidence Interval	0.195 - 0.219	0.201 - 0.225	0.109 - 0.359	0.02 - 0.44	0.135 - 0.324
	Adjusted P-Value	--	0.459			
	P-Value	--	0.156			
Furfural	Mean	<0.000100 ^a	<0.000100 ^a			
	Range	<0.000100 ^a	<0.000100 ^a			
	Confidence Interval	NA	NA	<0.000100 ^a	ND	<0.000100 ^a
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Inositol	Mean	0.0248	0.0236			
	Range	0.0175 - 0.0351	0.0160 - 0.0362			
	Confidence Interval	0.0215 - 0.0281	0.0204 - 0.0269	0.00684 - 0.0509	0.0063 - 0.48	0.0131 - 0.0344
	Adjusted P-Value	--	0.459			
	P-Value	--	0.113			
Phytic Acid	Mean	0.895	0.878			
	Range	0.500 - 1.27	0.456 - 1.24			
	Confidence Interval	0.762 - 1.03	0.744 - 1.01	0.516 - 1.37	ND - 1.940	<0.355 ^a - 1.34
	Adjusted P-Value	--	0.830			
	P-Value	--	0.559			
Raffinose	Mean	0.0995	0.104			
	Range	<0.0800 ^a - 0.183	<0.0800 ^a - 0.246			
	Confidence Interval	0.0651 - 0.134	0.0701 - 0.139	0 - 0.440	ND - 0.466	<0.0800 ^a - 0.301
	Adjusted P-Value	--	0.788			
	P-Value	--	0.440			
Trypsin Inhibitor (TIU/mg DW)	Mean	1.69	1.66			
	Range	1.22 - 3.25	1.05 - 2.83			
	Confidence Interval	1.55 - 1.83	1.52 - 1.80	1.02 - 5.68	ND - 8.42	1.03 - 3.01
	Adjusted P-Value	--	0.876			
	P-Value	--	0.735			

Note: Secondary metabolites and anti-nutrients unit of measure is % dry weight or as indicated. Trypsin inhibitors unit of measure is trypsin inhibitor units per milligram dry weight (TIU/mg DW). 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. Not applicable (NA): mixed model analysis was not performed or confidence interval was not determined.

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

Table 20. Number of Secondary Metabolites and Anti-Nutrients Sample Values Below the Lower Limit of Quantification for DP202216 Maize Grain

Analyte	Number of Samples Below the LLOQ	
	Control Maize (n=32)	DP202216 Maize (n=32)
Furfural	32	32
Raffinose ^a	12	9

Note: Secondary metabolites and anti-nutrients unit of measure is % dry weight.

^a This analyte had <50% below LLOQ sample values in DP202216 maize and was subjected to the mixed model analysis

VII-D. Conclusions on Compositional Assessment of DP202216 Maize

A compositional comparative assessment was conducted to determine if DP202216 maize grain 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 DP202216 maize is comparable to conventional (non-GE) maize.

The compositional analyses of grain 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 70 different analytical components (61 in grain, nine in forage) were presented and discussed. No statistical differences were observed in any of the analytes measured between DP202216 maize and its near-isoline control. Based on these analyses, the grain and forage of DP202216 maize are comparable to conventional maize with respect to nutrient composition.

Based on the results of the compositional evaluation, the grain and forage of DP202216 maize are as safe as conventional maize and is not expected to result in any significant impact on raw or processed maize products.

VIII. Agronomic Performance and Ecological Observations

Agronomic and ecological evaluations were conducted to assess the comparability of DP202216 maize to conventional maize. These evaluations form the basis to determine whether DP202216 maize is comparable to conventional maize and is therefore no more likely to pose a plant pest risk.

Agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of maize. To evaluate the agronomic characteristics of DP202216 maize, data were collected on representative characteristics that influence reproduction, crop survival, and potential weediness. In each of these assessments, DP202216 maize was compared to a near-isoline control that was >95% genetically similar to DP202216 maize but did not carry any recombinant DNA, and, in some experiments, was compared to non-genetically engineered conventional maize lines selected from current Pioneer conventional maize products. In each experiment, DP202216 maize was comparable to the control or conventional comparators.

The ecological evaluations included observed responses to insect and disease stressors during multi-year and multi-site field trials. These observations were made on DP202216 maize and control maize and tracked the presence of insect and disease stressors in the field and the plant responses. In each case, DP202216 maize responded similarly to the control plants in these trials.

Based on the analyses described below, DP202216 maize is comparable to conventional maize and would not 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 [] generation (Figure 2 and Table 1) of DP202216 maize were tested for germination assays under warm, cold, and diurnal conditions (Table 21). The [] generation of seed was used as [] hybrid seed is representative of seed that growers would plant in commercial maize fields. A near-isoline control was used for comparison. In addition, six non-GE conventional maize lines, (35P12, P0506, P0589, P0760, 34N84, and P0987), 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 DP202216 maize fell within this reference range, it indicated that DP202216 maize was comparable to conventional maize lines.

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Each germination test contained eight replicates of 50 seeds each of DP202216 maize, near-isoline control, and six conventional lines. The “International Rules for Seed Testing 2017”, published by the International Seed Testing Association, were used as guidelines for the

germination methods and interpretation of results (ISTA, 2017). Each replicate was placed between sheets of moist germination paper and rolled up with a piece of wax paper wrapped around the moist paper, and placed in a growth chamber set to the appropriate test conditions as specified in Table 21. 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 22. 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 23, 24, and 25.

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

Germination rates in DP202216 maize under warm, cold, and diurnal growing conditions were comparable to those of control maize under corresponding growing conditions.

The data provided here support the conclusion that DP202216 maize is comparable to conventional maize with respect to germination and viability.

Table 21. Description of Seed Germination Conditions

Warm Germination Test	<ul style="list-style-type: none"> • Continuous setting of 25°C and 90% relative humidity for 7 days • Evaluated after 7 days
Cold Germination Test	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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 22. 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/will 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 23. Summary of Warm Germination Test Results

Reported Statistic	DP202216 Maize	Control Maize	Reference Range
Frequency ^a	398/400	400/400	98.0% - 100%
Mean ^b	99.5%	100%	
Range ^b	98.0% - 100%	100% - 100%	
P-Value ^c	--	0.4994	

^a Total germination frequency across replicates.

^b Mean and range of germination rates for individual replicates.

^c P-value was determined using Fisher's exact test for germination rates.

Table 24. Summary of Cold Germination Test Results

Reported Statistic	DP202216 Maize	Control Maize	Reference Range
Frequency ^a	399/400	400/400	96.0% - 100%
Mean ^b	99.8%	100%	
Range ^b	98.0% - 100%	100% - 100%	
P-Value ^c	--	1.0000	

^a Total germination frequency across replicates.

^b Mean and range of germination rates for individual replicates.

^c P-value was determined using Fisher's exact test for germination rates.

Table 25. Summary of Diurnal Germination Test Results

Reported Statistic	DP202216 Maize	Control Maize	Reference Range
Frequency ^a	400/400	399/400	96.0% - 100%
Mean ^b	100%	99.8%	
Range ^b	100% - 100%	98.0% - 100%	
P-Value ^c	--	1.0000	

^a Total germination frequency across replicates.

^b Mean and range of germination rates for individual replicates.

^c P-value was determined using Fisher’s exact test for germination rates.

VIII-B. Field Trial Evaluations

VIII-B.1. Agronomic Data

Agronomic data were collected from the [] generation of DP202216 maize and near isoline control maize during the 2017 growing season at 12 sites in maize-growing regions of the United States (three sites in Iowa, two sites in Illinois, and one site in Indiana, Kansas, Missouri, Nebraska, Pennsylvania, and Texas) and Canada (one site in Ontario). Figure 20 and Table 26 provide more information regarding field trial locations. The [] generation of seed was used as [] hybrid seed is representative of seed that growers would plant in production maize fields. The trial locations provided a range of environmental and agronomic conditions representative of the major maize growing regions of the United States and Canada, where production of DP202216 maize is expected. Agronomic parameters observed are provided in Table 27.

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Agronomic characteristics of DP202216 maize were evaluated in comparison to concurrently grown non-GE, near-isoline maize (referred to as control maize) to identify statistical differences, and subsequently were evaluated in the context of normal ranges of variation established from concurrently grown non-GE, conventional maize (referred to as reference maize) data.

Evaluation of agronomic characteristics of DP202216 maize included early stand count, days to flowering, height, lodging, final stand count, days to maturity, pollen viability, kernels per ear (calculated from kernel rows per ear and kernels per row), harvest grain moisture, yield, and 100-kernel weight.

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 DP202216 maize to conventional maize are appropriate.

A randomized complete block design with four blocks was utilized at each site. Each block included DP202216 maize, non-genetically engineered (non-GE) near-isoline control maize (referred to as control maize), and four of the following non-GE conventional maize lines: 34N84, 35F38, 35P12, P0506, P0589, P0760, P0965, P0987, P0993, XL5140, XL5513, XL5828, XL5840, BK5883, XL5939, and BK6076 (referred to as reference maize). These conventional products were chosen to represent a range of non-genetically engineered hybrids that are planted commercially. Agronomic data collected from the reference maize were used to help determine the normal range of variation for the agronomic characteristics in conventional maize.

Details of the methods used are presented in Appendix 12.

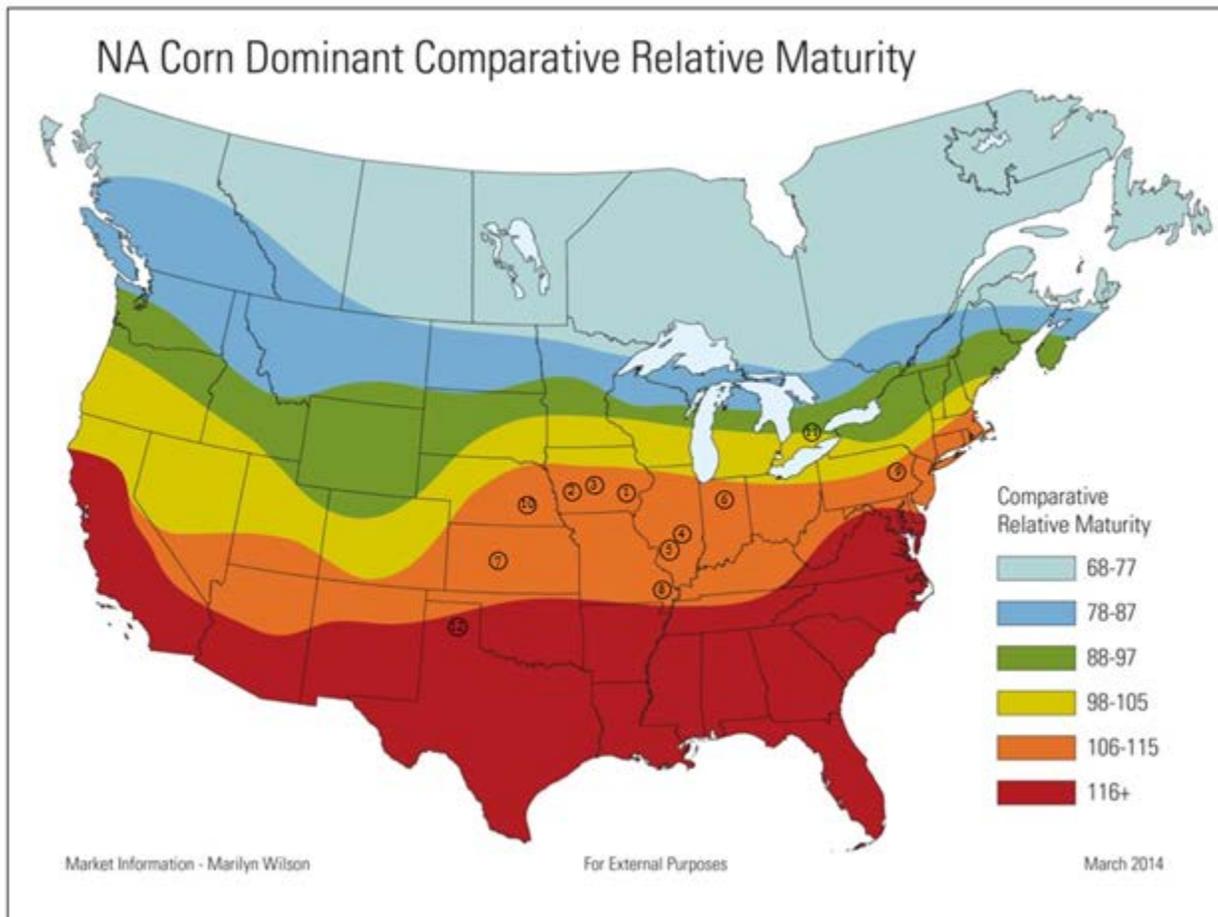


Figure 20. Distribution of Field Location – 2017 DP202216 Maize Field Trials

Reference	Site Code	Site Location
1	RG005IA1	Richland, Iowa, USA
2	RG005IA5	Atlantic, Iowa, USA
3	RG005IA7	Johnston, Iowa, USA
4	RG005IL5	Stewardson, Illinois, USA
5	RG005IL7	Carlyle, Illinois, USA
6	RG005IN2	Sheridan, Indiana, USA
7	RG005KS1	Larned, Kansas, USA
8	RG005MO5	Fisk, Missouri, USA
9	RG005PA1	Germansville, Pennsylvania, USA
10	RG005NE5	York, Nebraska, USA
11	RG005ON3	Guelph, Ontario, Canada
12	RG005TX7	Groom, Texas, USA

Table 26. Location Information – 2017 DP202216 Maize Field Trials

Agronomic characteristic evaluation results are provided in Table 28. Details regarding statistical analysis methods are provided in Appendix 12.

A total of 20 agronomic endpoints were included in the assessment: 16 were evaluated using mixed model analysis; one agronomic characteristic was evaluated using the generalized Cochran-Mantel-Haenszel (CMH) test (lodging). The remaining three agronomic characteristics (pollen viability-shape at 60 and 120 minutes and pollen viability-color at 120 minutes) exceeded criteria for maximum number of uniform values and were therefore not subjected to comparative analyses.

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 DP202216 maize was compared to the in-study reference range comprised of all individual values across-sites from all non-GE reference maize lines grown in this study. In cases when a raw P-value indicated a significant difference but the False Discovery Rate (FDR) adjusted P-value was > 0.05, it was concluded that the difference was likely a false positive.

DP202216 maize contains a trait which results in an enhanced grain yield response across a wide range of genotypes and environments. The 2017 regulatory science agronomic characteristic study did not reveal a statistical difference in yield in comparison with the non-GE near-isoline control maize. This agronomic regulatory study was statistically designed to evaluate and assess the safety of the DP202216 event across a representative and diverse set of environments for a single genotype in a single year. As such, it would not have been expected to show a statistical difference in yield in the agronomic regulatory study. In product development research studies conducted under field permit, agronomics and grain yield were evaluated across a larger number of genotypes with a significantly larger number of replications involving multiple years and sites. These product development studies are designed to detect small but economically valuable yield

difference in environments that closely mimic the competitive effects found in grower farm fields. Although the yield effects of DP202216 maize are consistently positive on average, as one would expect, within these large data sets there are instances where the trait does not demonstrate a statistically significant grain yield improvement for individual environments or genotypes. Thus, the lack of yield improvement in the 2017 regulatory science agronomic characteristic study is consistent with the commercial development data.

Table 27. Agronomic Characteristics Measured

Characteristic	Evaluation Timing^a	Description	Scale
Early Stand Count	V2-V4	Total number of plants emerged per plot	Numerical Count per meter squared
Days to Flowering	50% of plants shedding pollen	From the time of planting until approximately 50% of plants have tassels shedding pollen	Days
Plant Height	R4	Height from soil surface to collar of flag leaf	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	Numerical Count
Days to Maturity	Physiological maturity	Number of days for majority of plants to first reach physiological maturity	Days
Pollen Viability ^b	During active pollen shed	Shape and color at 0, 30, 60, and 120 minutes	Percent of grains with collapsed walls and yellow color
Kernel Rows per Ear	Post-harvest	Total number of kernels rows per ear for 5 primary ears	Average of numerical count
Kernels per Row	Post-harvest	Total number of kernels per row (4 rows counted) for 5 primary ears	Numerical count
Kernels per Ear	Post-harvest	Kernel rows per ear multiplied by average number of kernels per row	Calculated
Harvest Grain Moisture	Approximately R6	Moisture content of harvested grain	Percent
Yield	Approximately R6	Harvest weight per area adjusted to 15.5% moisture	Calculated bushels per acre
100 Kernel Weight	R6	Total weight of 100 kernels of pooled grain, adjusted 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 28. Across-Site Analysis of Agronomic Characteristics Results

Agronomic Characteristic	Reported Statistics	Control Maize	DP202216 Maize	Reference Data Range
Early Stand (count/m ²)	Mean	6.2	6.2	
	Range	5.7 - 6.9	5.1 - 6.8	
	Confidence Interval	6.1 - 6.4	6.0 - 6.3	4.6 - 6.6
	Adjusted P-Value	--	0.272	
	P-Value	--	0.0540	
Days to Flowering (days)	Mean	62.6	62.7	
	Range	54 - 72	55 - 74	
	Confidence Interval	59.5 - 65.8	59.6 - 65.9	53 - 74
	Adjusted P-Value	--	0.866	
	P-Value	--	0.601	
Pollen Viability-Shape, 0 minutes (% of pollen with collapsed walls)	Mean	7.4	6.4	
	Range	0 - 60	0 - 50	
	Confidence Interval	1.9 - 16.1	1.4 - 14.7	0 - 50
	Adjusted P-Value	--	0.640	
	P-Value	--	0.377	
Pollen Viability-Shape, 30 minutes (% of pollen with collapsed walls)	Mean	58.5	58.7	
	Range	5 - 100	10 - 100	
	Confidence Interval	38.9 - 78.2	39.0 - 78.4	5 - 100
	Adjusted P-Value	--	0.921	
	P-Value	--	0.921	
Pollen Viability-Shape, 60 minutes (% of pollen with collapsed walls)	Mean	86.5	89.1	
	Range	20 - 100	25 - 100	
	Confidence Interval	NA	NA	20 - 100
	Adjusted P-Value	--	NA	
	P-Value	--	NA	
Pollen Viability-Shape, 120 minutes (% of pollen with collapsed walls)	Mean	96.6	96.9	
	Range	65 - 100	60 - 100	
	Confidence Interval	NA	NA	60 - 100
	Adjusted P-Value	--	NA	
	P-Value	--	NA	
Pollen Viability-Color, 0 minutes (% of pollen yellow in color)	Mean	8.7	7.4	
	Range	0 - 60	0 - 60	
	Confidence Interval	2.6 - 17.9	1.9 - 16.0	0 - 60
	Adjusted P-Value	--	0.640	
	P-Value	--	0.355	
Pollen Viability-Color, 30 minutes (% of pollen yellow in color)	Mean	58.6	58.1	
	Range	5 - 100	5 - 100	
	Confidence Interval	39.7 - 77.6	39.1 - 77.1	5 - 100
	Adjusted P-Value	--	0.888	
	P-Value	--	0.783	
Pollen Viability-Color, 60 minutes (% of pollen yellow in color)	Mean	83.4	86.8	
	Range	20 - 100	20 - 100	
	Confidence Interval	70.6 - 96.3	73.9 - 99.6	10 - 100
	Adjusted P-Value	--	0.552	
	P-Value	--	0.195	
Pollen Viability-Color, 120 minutes (% of pollen yellow in color)	Mean	95.0	94.9	
	Range	45 - 100	40 - 100	
	Confidence Interval	NA	NA	40 - 100
	Adjusted P-Value	--	NA	
	P-Value	--	NA	

Table 28. Across-Site Analysis of Agronomic Characteristics Results (continued)

Agronomic Characteristic	Reported Statistics	Control Maize	DP202216 Maize	Reference Data Range
Plant Height (cm)	Mean	224.5	223.0	
	Range	171.0 - 279.4	169.2 - 287.6	
	Confidence Interval	208.0 - 240.9	206.6 - 239.5	170.0 - 311.8
	Adjusted P-Value	--	0.640	
	P-Value	--	0.294	
Days to Maturity (days)	Mean	130.8	129.6	
	Range	111 - 164	111 - 168	
	Confidence Interval	122.6 - 139.0	121.3 - 137.8	114 - 164
	Adjusted P-Value	--	0.272	
	P-Value	--	0.0686	
Lodging (%)	Mean	1.1	1.5	
	Range	0.0 - 8.5	0.0 - 12.4	
	Confidence Interval	NA	NA	0.0 - 16.2
	Adjusted P-Value	--	0.272	
	P-Value	--	0.0799	
Final Stand Count (count/m ²)	Mean	6.2	6.0	
	Range	5.7 - 6.5	5.1 - 6.6	
	Confidence Interval	6.0 - 6.3	5.9 - 6.2	4.8 - 6.5
	Adjusted P-Value	--	0.0519	
	P-Value	--	0.00611*	
Number of Kernel Rows per Ear	Mean	16.8	16.7	
	Range	14 - 18	15 - 18	
	Confidence Interval	16.4 - 17.2	16.3 - 17.1	12 - 20
	Adjusted P-Value	--	0.866	
	P-Value	--	0.614	
Average Number of Kernels Per Row	Mean	37.3	37.5	
	Range	28 - 43	31 - 43	
	Confidence Interval	35.4 - 39.1	35.6 - 39.3	29 - 47
	Adjusted P-Value	--	0.866	
	P-Value	--	0.713	
Number of Kernels Per Ear	Mean	625.1	626.0	
	Range	464 - 731	488 - 752	
	Confidence Interval	587.4 - 662.8	588.2 - 663.7	435 - 782
	Adjusted P-Value	--	0.921	
	P-Value	--	0.904	
Harvest Grain Moisture (%)	Mean	17.2	17.2	
	Range	10.2 - 23.3	10.2 - 23.7	
	Confidence Interval	15.2 - 19.3	15.2 - 19.3	10.5 - 27.2
	Adjusted P-Value	--	0.866	
	P-Value	--	0.681	
Yield (bu/A)	Mean	201.7	193.7	
	Range	117 - 272	85 - 261	
	Confidence Interval	176.9 - 226.5	168.9 - 218.5	102 - 292
	Adjusted P-Value	--	0.0519	
	P-Value	--	0.00563*	
100-Kernel Weight (g)	Mean	36.3	35.8	
	Range	25.7 - 41.9	25.9 - 43.4	
	Confidence Interval	34.0 - 38.6	33.5 - 38.1	22.9 - 45.9
	Adjusted P-Value	--	0.640	
	P-Value	--	0.336	

Note: Not applicable (NA); mixed model analysis was not performed.

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

VIII-B.2. Yield Evaluation of DP202216 and Control Maize

A series of grain yield field trials were conducted over 3 years (2014 – 2016) in North America to test yield performance of DP202216 maize. Yield evaluations were conducted across a diverse range of environmental conditions. All testing sites included near-isoline control hybrids and corresponding DP202216 maize hybrids. Each testing site utilized a split plot design with two to three replicates, with hybrid as the main plot and entry (control maize, DP202216 maize) as the sub plot. Yield data were collected at harvest. At some testing sites, irrigation was applied to achieve a range of yield levels representative of maize growing environments.

Grain weights and moisture levels for each experimental entry were measured by harvesting the center two rows of the four-row plot using a small-plot combine. Yield was standardized within the experiment by adjusting the harvested grain weight of each plot to fifteen percent moisture. A linear mixed model analysis was conducted for the combined three-year grain yield dataset, accounting for the experimental design of the multi-environmental trials. Yield mean values were estimated for DP202216 maize and control maize across all hybrids and across all environments combined across all three years as well as by individual years. The DP202216 maize yield mean values were compared to control maize mean values to test for significant yield differences. A statistically significant difference was identified when P-Value < 0.05

Statistical analysis results are provided in Table 29. A positive grain yield response was observed in DP202216 maize compared to the control maize across years, sites, and hybrids. DP202216 maize had increased grain yield by 3.4 bushels/acre across the combined years, compared to control maize. The positive grain yield response was consistently observed within each individual year, as well as across the combined years, and all differences were statistically significant (P-value < 0.05).

Table 29. Yield Comparison of DP202216 Maize to Control Maize Across Three Years of Testing

Year	N	Average Grain Yield (bushels/acre)		Yield Difference (bushels/acre) (95% CI)	P-Value
		DP202216 Maize	Control Maize		
2014	185	212.7	206.4	6.3 (3.7, 8.9)	<0.0001*
2015	302	200.4	198.1	2.3 (0.5, 4.1)	0.0129*
2016	331	208.6	205.9	2.7 (0.6, 4.9)	0.0148*
Combined	818	206.7	203.3	3.4 (2.1, 4.8)	<0.0001*

VIII-B.3. Biotic and Abiotic Observations of DP202216 and Control Maize

DP202216 maize has been evaluated for response to biotic and abiotic stressors in field tests located in the United States, United States territories, and Canada.

Experiment A – 2017 Field Trial Biotic and Abiotic Stressor Measurement

Data were collected from 12 sites in conventional maize-growing regions of the United States (three sites in Iowa, two sites in Illinois, and one site in Indiana, Kansas, Missouri, Nebraska, Pennsylvania, and Texas) and Canada (one site in Ontario) during the 2017 growing season. For each trial site, a survey of the naturally occurring insects, diseases, and abiotic stressors and any unexpected differences in the response of DP202216 maize as compared to the near-isoline control line, and conventional reference lines were recorded at four observation periods. These observations provide a means to determine if DP202216 maize will respond differently from conventional maize lines to insects, diseases, abiotic stressors in the environment.

Observations from field trials demonstrated that DP202216 maize did not exhibit any unexpected responses to naturally occurring insects or diseases, and abiotic stressors as summarized in Appendix 13. These results support the conclusion that DP202216 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 – 2009-2017 Field Observation Data

DP202216 maize has been field tested in the United States and Puerto Rico over 9 years, as authorized by USDA-APHIS permits and notifications. For each trial, a survey of the naturally occurring insects and diseases and any unexpected differences in the response of DP202216 maize as compared to the control line (near-isoline and/or conventional maize lines) were recorded by experienced plant breeders and field staff at least every four weeks. A summary of these surveys for each trial and any differences seen between DP202216 maize and control lines are presented in Appendix 13. These observations provide a means to determine if DP202216 maize will respond differently from conventional maize lines to insects or diseases in the environment.

In every case, DP202216 maize did not exhibit any unexpected responses to naturally occurring insects or diseases. These results, taken with the results presented above, support the conclusion that DP202216 maize is comparable to control maize lines with similar genetics or to conventional maize lines with respect to insect or disease response.

VIII-C. Conclusions on Agronomic Performance and Field Observations of DP202216

Maize

DP202216 maize was observed in laboratory experiments and at 12 field locations in the United States and Canada to measure agronomic parameters and abiotic and biotic stressors. These experiments and field studies evaluate the characteristics of maize over a broad range of environmental conditions that represent regions where DP202216 maize will be grown. The agronomic parameters measured are characteristic traits for reproduction, survival, and potential weediness.

The agronomic data from Experiment A demonstrated no significant differences between DP202216 maize and control maize (near-isoline controls and/or conventional maize lines) with respect to early population, vegetative growth, reproductive parameters, yield, and pest responses. These data support the conclusion that DP202216 maize is agronomically comparable to conventional maize.

Observations from United States and United States territory field trials (Experiment B) over multiple years showed no unexpected differences in the response of DP202216 maize and control maize to naturally occurring insects and diseases. These results support the conclusion that DP202216 maize is comparable to control maize lines with similar genetics and/or to conventional maize lines.

Based on these analyses, DP202216 maize is comparable to conventional maize and will not pose a greater plant pest risk or increased weed potential than conventional maize.

IX. Potential Environmental Impact of the Introduction of DP202216 Maize

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

To evaluate the potential environmental impact of the introduction of DP202216 maize, the potential for DP202216 maize to become weedy or invasive, the potential for gene flow to sexually compatible wild relatives, and the potential impacts of the introduced proteins (ZMM28

and PAT) were considered. As described further below, in each case, it is not expected that DP202216 maize will adversely impact the environment with respect to these considerations.

IX-A. Potential for DP202216 Maize to Have Altered Disease and Unintended Pest Susceptibilities or to Become Weedy or Invasive

In evaluating the potential for DP202216 maize to become more weedy or invasive than conventional maize, general maize biology was considered. Maize is a cultivated annual plant that generally cannot survive temperatures below freezing and is typically grown in temperate regions (OECD, 2003). Maize is not classified as a weed, is not on the United States federal or state noxious weed lists, and possesses few characteristics of notably successful weeds (Baker, 1974; Keeler, 1989; USDA-NRCS, 2011). Therefore, the natural characteristics of maize do not indicate a high potential for weediness or invasiveness.

A comparative assessment of DP202216 maize was conducted to determine if the DNA insertion altered the nutritive or agronomic characteristics of maize. Composition and agronomic comparison data were collected on DP202216 maize in multiple location field trials as described in Sections VIII and VII, respectively. These analyses showed that DP202216 maize was comparable to conventional maize in composition, and was comparable in agronomics. In the agronomic analyses, plant characteristics were measured, including certain ones that may be indicative of weediness: germination and emergence (germination rate, early stand count); reproductive characteristics (days to flowering, days to maturity, pollen viability, kernels per ear, and kernels per row, yield, and 100-kernel weight); vegetative characteristics (final stand count, 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. DP202216 maize was comparable to conventional maize in each of these characteristics, indicating that DP202216 maize is unlikely to become more weedy or invasive than conventional maize.

In addition, DP202216 maize has been field tested since 2009 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 DP202216 maize and control plants. In all cases, no unexpected differences were observed between DP202216 maize and the control comparators.

Extensive nutrient composition analyses of grain and forage were conducted to compare the composition of DP202216 maize to controls. These analyses were used to evaluate any changes

in the levels of key nutrients, anti-nutrients and secondary metabolites. Based on the results of the compositional evaluation, the grain and forage of DP202216 maize are as safe as conventional maize and is not expected to result in any significant impacts on raw or processed maize commodities.

In summary, DP202216 maize is unlikely to become more weedy or invasive than conventional maize when cultivated. Compositional and agronomic comparisons indicate no unexpected effects of the presence of the introduced proteins that alter the nutritional composition and weediness potential of maize. No unexpected differences were detected between DP202216 maize and control maize in response to insects and diseases. Furthermore, the expression of the introduced proteins (ZMM28 and PAT) is unlikely to increase the potential of DP202216 maize to become weedy.

IX-B. Potential for Gene Flow Between DP202216 Maize and Sexually Compatible Wild Relatives

The potential for gene flow between a genetically engineered 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 genetically engineered 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.

DP202216 maize will 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 DP202216 maize and its wild relatives of the genera *Zea* and *Tripsacum* in the United States.

The potential for the insertion in DP202216 maize to become widespread in wild relative populations is also unlikely. The insertion, as discussed in Section IV, does not make DP202216 maize 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 will travel significantly outside of the cultivated field. Pollen viability is reduced in a matter of hours under high temperature and low humidity (Aylor, 2004). Studies also indicate that the majority of maize pollen is unlikely to be dispersed significant distances outside the originating field (Jarosz et al., 2003). Numerous studies show the majority (84-92%) of pollen grains travel less than five meters (Pleasant 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 will 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 DP202216 maize and conventional maize in pollen viability via measurements of shape and color over time. Pollen viability of DP202216 maize 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 21 indicates acres planted in the United States by county (USDA-NASS, 2011).

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

It is expected that DP202216 maize will be cultivated in the same maize production regions as conventional maize.

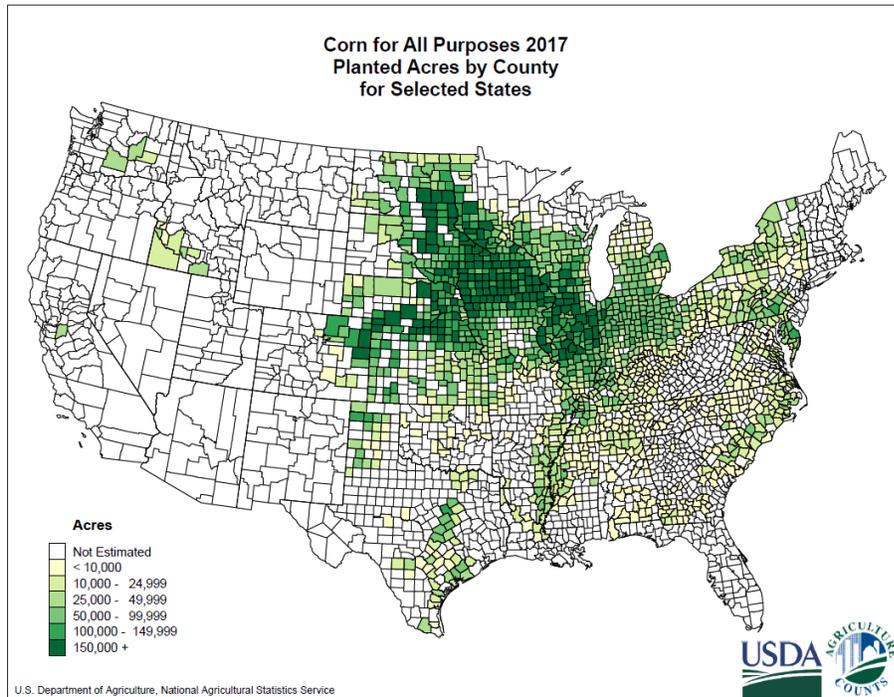


Figure 21. 2017 Corn Planted Acres - USDA-NASS (2017)

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 U.S., sparsely dispersed introduced populations of annual teosintes *Zea mexicana* (synonym: *Zea mays* ssp. *mexicana*)

and *Zea mays* ssp. *parviglumis* have been reported in Florida, Maryland, and Alabama (USDA, 2011). Also, an isolated population of *Zea perennis* (perennial teosinte) has been introduced in South Carolina (USDA, 2011). While maize can hybridize with these species under natural conditions, there is incompatibility between some maize populations and certain types of teosinte that results in low fitness of some hybrids and prevents a high rate of introgression (OECD, 2003). Together with the very limited geographic range of the teosinte population in the U.S., the probability of gene flow from cultivated maize fields to these wild relatives is very low.

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

Conclusions on the Potential for Gene Flow between DP202216 Maize 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 DP202216 maize does not exhibit greater potential for weediness as determined from agronomic comparisons to conventional maize. Therefore, any incidental gene flow between DP202216 maize and its wild relatives would not transform maize wild relatives into more weedy species, nor would the introduced trait be introgressed widely in wild relative populations.

X. Adverse Consequences of Introduction

The data and information presented in this petition demonstrate that DP202216 maize is unlikely to pose a plant pest risk as compared to conventional maize. The analysis of molecular data confirmed the insertion of one copy of the PHP40099 T-DNA, containing the ZMM28 and PAT expression cassettes. The PHP40099 insert is stably integrated at a single locus and follows Mendelian inheritance principles over 5 breeding generations.

The analysis of nutrients, anti-nutrients, and secondary metabolites of DP202216 maize demonstrates the compositional equivalence of DP202216 maize to conventional maize. Evaluation of seed germination and dormancy characteristics showed equivalency to conventional maize controls. Agronomic characteristics inclusive of plant growth and development, reproductive, and vegetative parameters indicated no change, except for enhanced yield potential in multi-year, broad acreage field trials, when compared to conventional maize.

Measurement of response to biotic and abiotic stressors, insects, or disease also show no difference when compared to conventional maize. This dataset implies no indication that DP202216 maize would have an adverse impact on non-target or beneficial organisms, as well as endangered or threatened species.

Introduction of DP202216 maize will not 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 DP202216 maize does not present a plant pest risk and is not otherwise deleterious to the environment. Therefore, Pioneer requests that APHIS grant the request for a determination of nonregulated status for DP202216 maize, DP202216 maize progeny, and any crosses of DP202216 maize with other nonregulated maize.

Appendices

Appendix 1.

DP202216 Maize USDA Release Permits, Notifications, and Planted Acreage

Year of Planting	Permit Name	Permit Valid Date	State	Number of Counties Where DP-202216-6 Maize was Planted	Acreage
2009	09-033-112n	2/24/2009	HI	1	0.001
			PR	1	0.001
	09-264-101n	10/6/2009	PR	1	0.090
2010	09-264-101n	10/6/2009	HI	1	0.003
			PR	1	0.004
	10-052-101n	3/19/2010	HI	1	0.006
			PR	1	0.003
			IA	1	0.004
10-284-101n	11/16/2010	HI	1	0.001	
2011	11-040-122n	3/17/2011	HI	1	0.012
			IA	1	0.005
	11-288-101n	11/16/2011	HI	1	0.002
			PR	2	0.140
2012	12-011-102n	2/13/2012	HI	1	0.005
	12-202-101n	8/15/2012	HI	1	0.002
			PR	1	0.021
2013	12-202-101n	8/15/2012	HI	1	0.002
			HI	1	0.029
	13-009-102n	2/21/2013	PR	1	0.027
			HI	1	0.012
	13-294-102n	11/12/2013	PR	1	0.073
AR			1	0.042	
2014	14-002-104n	1/30/2014	CA	1	0.120
			HI	1	0.036
			IL	3	0.205
			IN	1	0.042
			IA	4	0.278
			NE	1	0.063
			TN	1	0.009
	14-300-106n	11/12/2014	HI	1	0.057
			PR	1	0.151

DP202216 Maize USDA Release Permits and Notifications and Planted Acreage (continued)

Year of Planting	Permit Name	Permit Valid Date	State	Number of Counties Where DP-202216-6 Maize was Planted	Acreage	
2015	15-012-101n	2/18/2015	CA	1	0.063	
			HI	1	0.002	
			IL	4	0.137	
			IN	1	0.026	
			IA	5	0.177	
			KS	1	0.005	
			MO	1	0.012	
			NE	2	0.028	
			PR	2	0.113	
			TX	1	0.005	
				WI	1	0.001
		15-300-101n	12/1/2015	HI	1	0.104
PR	1			0.068		
2016	16-015-103n	2/22/2016	CA	1	0.722	
			HI	1	0.062	
			IL	4	0.384	
			IN	1	0.004	
			IA	3	0.424	
			KS	2	0.228	
			MN	1	0.002	
			NE	1	0.002	
			SD	1	0.002	
			TX	1	0.226	
				WI	1	0.001
		16-291-102n	11/7/2016	HI	1	0.107
	PR			1	0.928	
		16-291-102n	11/7/2016	PR	2	0.120

DP202216 Maize USDA Release Permits and Notifications and Planted Acreage (continued)

Year of Planting	Permit Name	Permit Valid Date	State	Number of Counties Where DP-202216-6 Maize was Planted	Acreage
2017	17-017-102n	2/1/2017	CA	1	0.900
			HI	1	0.087
			IL	3	0.084
			IN	1	0.024
			IA	3	0.760
			KS	2	0.195
			NE	1	0.024
			PR	2	0.555
			TN	1	0.020
			TX	1	0.037
	WI	1	0.003		
	17-019-101n	2/28/2017	IL	2	0.069
			IN	1	0.034
			IA	2	0.069
			KS	1	0.034
			MO	1	0.034
			NE	1	0.091
PA			1	0.034	
TX	1	0.034			
17-048-106n	3/6/2017	IL	1	0.057	
17-285-103n	11/2/2017	HI	1	0.419	
17-311-102n	11/17/2017	PR	1	0.159	
2018	18-016-102n	2/15/2018	IL	1	0.203
			NE	1	0.203
	18-016-103n	2/12/2018	CA	1	0.040
			IA	2	0.029
			NE	1	0.016
	18-033-103rm	4/5/2018	CA	1	0.050
			IL	3	0.320
			IN	1	0.294
			IA	4	0.948
			KS	1	0.030
MO			1	0.012	
NE			2	0.324	
TN	1	0.012			
TX	1	0.030			

Appendix 2. Methods and Results for Southern by Sequencing Analysis

Test Material

Seeds from the [] generation of DP202216 maize (referred to as DP202216 maize) were planted, and leaf tissue harvested.

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Control Material

Seeds from a non-GE (conventional) maize line, [], were planted and leaf tissue harvested from individual plants was used in genomic DNA extraction.

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Reference Material

Plasmid dilutions for SbS analysis were prepared from plasmid PHP40099 (Figure 1) that was used for *Agrobacterium*-mediated transformation to produce DP202216 maize.

Plant Growth and Sample Collection

Test and control substance (DP202216 maize and control maize) seeds were planted and grown and leaf tissue was collected as part of study PHI-2017-047 (Kallal and TeRonde, 2018). 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, all plants were analyzed by polymerase chain reaction (PCR) as part of study PHI-2017-047 (Kallal and TeRonde, 2018). Control maize plants were tested for the absence of the *zmm28* and *mo-pat* genes, while DP202216 maize plants were tested with an event-specific assay for the DP202216 insertion as well as both gene-specific assays. Control maize plants were negative for all assays. Of the eight DP202216 maize plants, six were positive and thus contained the inserted PHP40099 T-DNA, while two were negative for all assays, indicating they did not contain the insertion (negative plants).

DNA Extraction and Quantitation

Genomic DNA was extracted from leaf tissue of DP202216 and control maize plants. The tissue was lyophilized and pulverized in tubes using a Geno/Grinder™ (SPEX CertiPrep, Inc., Metuchen, NJ) instrument. Genomic DNA was isolated using Cetyltrimethylammonium bromide extraction buffer followed by purification with a Genomic-tip 100/G column (QIAGEN, Valencia, CA). Following extraction, the DNA was quantified on a spectrofluorometer using PicoGreen® reagent

(Molecular Probes, Inc., Eugene, OR) and visualized on an agarose gel to confirm values from PicoGreen analysis and to determine the DNA quality (Figure 5, Step 1).

Southern-by-Sequencing

SbS was performed by Pioneer Analytical and Genomics Technologies (Johnston, IA). SbS analysis utilizes probe-based sequence capture, Next Generation Sequencing (NGS) techniques, and bioinformatics procedures to capture, sequence, and identify inserted DNA within the maize genome (Zastrow-Hayes et al., 2015). By compiling a large number of unique sequencing reads and mapping them against the linearized transformation plasmid 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, insertion intactness, and to confirm the absence of plasmid backbone sequences. Eight plants of the [] generation of DP202216 maize were analyzed by SbS to determine the insertion copy number and intactness in each plant. Six plants contained the DP202216 DNA insertion as shown by event-specific PCR analysis; the remaining two plants were shown to be negative for the insertion by the same assay. SbS was also performed on a positive control sample (control maize DNA spiked with PHP40099 plasmid at a level corresponding to one copy of PHP40099 per copy of the maize genome) to confirm that the assay could reliably detect plasmid fragments within the genomic DNA.

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The following processes were performed by Pioneer Analytical and 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 PHP40099 plasmid sequences were designed and synthesized by Roche NimbleGen, Inc. (Madison, WI). The probe set was designed to target all sequences within the PHP40099 transformation plasmid (Figure 5, Step 2).

Sequencing Library Construction

Next generation sequencing (NGS) libraries were constructed for DNA samples from individual DP202216 maize plants, 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., Austin, TX) 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 5, 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 5, 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 5, 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 5, 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 PHP40099 T-DNA sequence using Bowtie with zero mismatches allowed. Any “Non-redundantReads” that were not wholly derived from either sequence were aligned to the

PHP40099 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 PHP40099 plasmid backbone sequences in the genomic DNA samples due to similarity with the PHP40099 backbone region. “Non-redundantReads” that aligned to the PHP40099 backbone sequence, but at a coverage depth below 35x across 50 bp, were deemed to be due to environmental bacteria (Figure 5, 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 PHP40099 plasmid sequence using the Burrows-Wheeler Aligner (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 PHP40099 sequence from the alignment, such as plasmid-to-genome junctions or rearrangements of the plasmid. These chimeric reads are referred to as junction reads or junctions. The individual reads defining a junction were condensed to a unique identifier to represent the junction. This identifier (referred to as a 30_20 mer) includes 20 bp of sequence from PHP40099 and 30 bp of sequence adjacent to the 20 bp from the plasmid. The adjacent 30 bp did not align to PHP40099 contiguously to the known 20 bp. When the 20 bp from PHP40099 and the adjacent 30 bp are combined into a 30_20 mer, they indicate the junction shown by the chimeric read. Junction reads were condensed into a unique junction if their 30_20 mers were identical, or if the 30_20 mer junctions were within 2 bp. The total number of sequence reads (referred to as “TotalSupportingReads”) for each unique junction was retained for filtering. Junctions with fewer than five unique supporting reads, or if the “TotalSupportingReads” value was below 10% of the median sequencing depth for positions aligned to the plasmid, were filtered and removed from further analysis (Figure 5, Step 8).

Junction Identification

Variations between the maize reference genome and the sequences of endogenous maize sequences that are found in the transformation construct may result in identification of junctions that are due to these endogenous maize sequences. To detect these endogenous junctions, control maize genomic DNA libraries were captured and sequenced in the same manner. These libraries were sequenced to an average depth approximately five times that of the depth for the DP202216 maize plant samples. This increased the probability that the endogenous junctions captured by the PHP40099 probes would be detected in the control maize samples, so that they

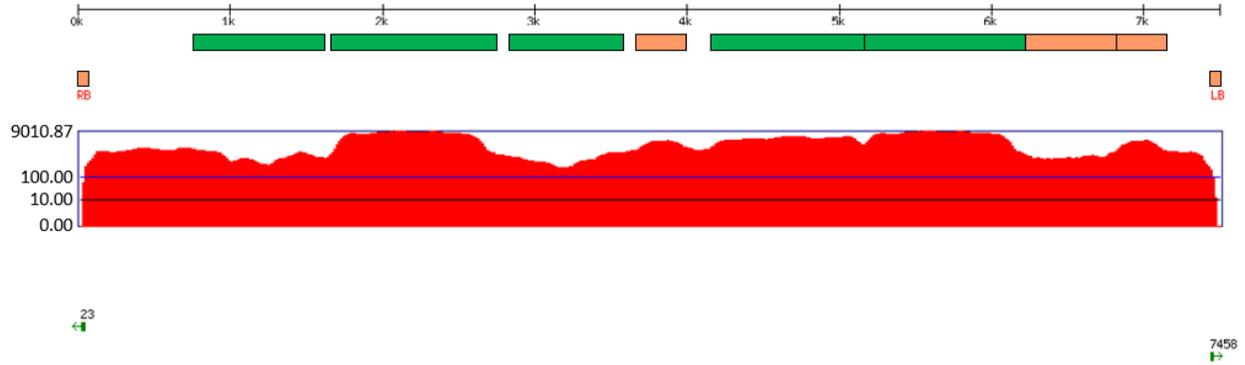
could be identified and removed from the DP202216 maize samples. The 30_20 mers of the endogenous junctions detected in this analysis were used to filter the same endogenous junctions in the DP202216 maize samples (Figure 5, Step 8), so that the only junctions remaining in the DP202216 samples are due to actual PHP40099 insertions (Figure 5, Step 9).

SbS Results

Results for the control maize, positive control, and “representative plant” (Plant ID 335728647) are presented in the main body (Part V.B.) of this document.

Remaining plant results from SbS analysis follow:

A.



B.

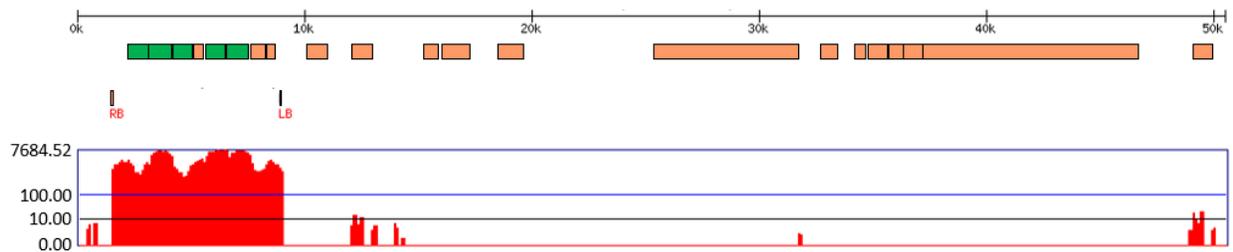
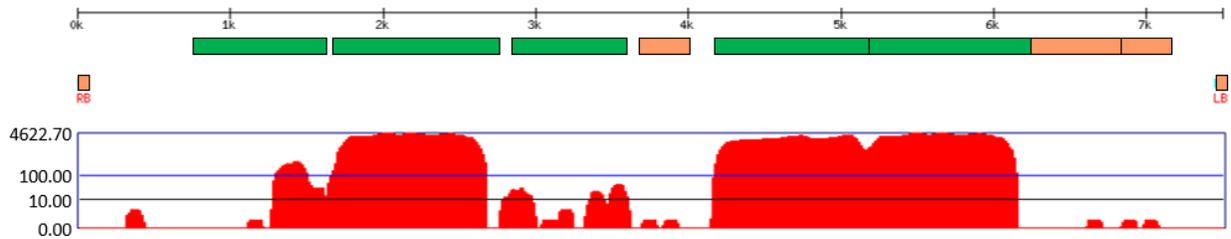


Figure A2-1. SbS Results for DP202216 Maize (Plant ID 335728648)

This sample was positive for the DP202216 insertion as confirmed by PCR. 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 indicates endogenous genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Dark green arrows in the Junctions section show 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 intact T-DNA sequence. The insertion comprises bp 23 to 7,458 of the PHP40099 T-DNA shown in Figure 2. The presence of only two junctions when aligned to the T-DNA demonstrates the presence of a single PHP40099 T-DNA in the DP202216 maize genome. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the T-DNA region near the left of the coverage graph; however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP40099 sequence shows that there are no additional insertions or backbone sequence present in DP202216 maize.

A.



B.

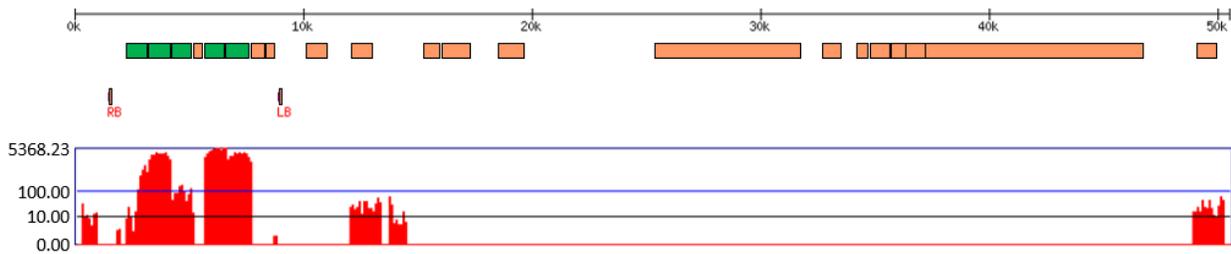
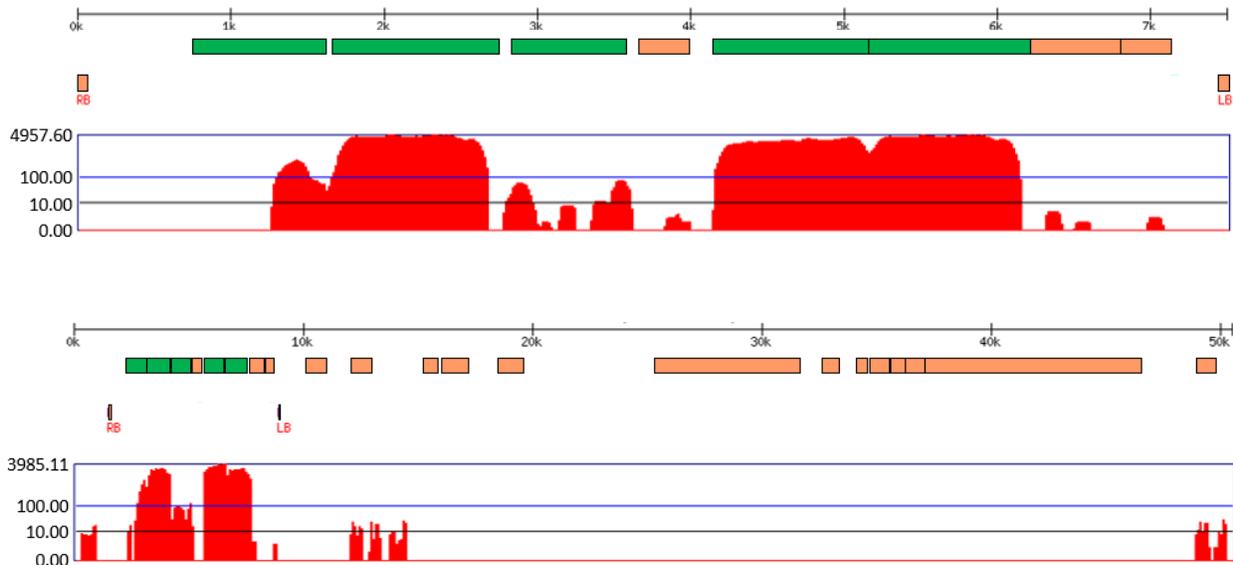


Figure A2-2. SbS Results for DP202216 Maize (Plant ID 335728649)

This sample was negative for the DP202216 insertion as confirmed by PCR. 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 indicates endogenous genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Coverage was obtained to the same maize endogenous elements as in the [] control maize but the lack of junctions to genomic DNA indicates that the coverage is to the elements in their normal genomic context and are not related to any insertion in this plant. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the endogenous maize elements in the T-DNA region near the left of the coverage graph; however, no junctions to PHP40099 sequences were identified. The absence of any junctions to the PHP40099 sequence shows that there are no insertions or backbone sequence present in this plant.

A.

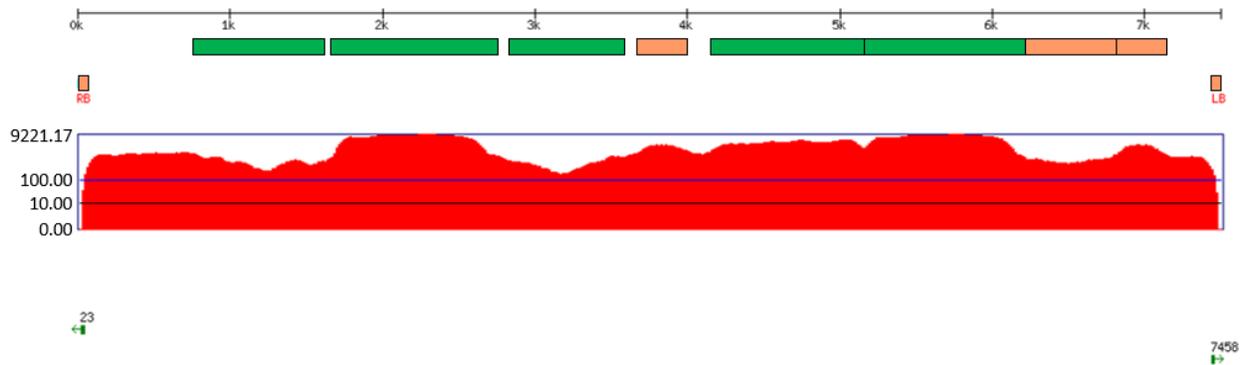


B.

Figure A2-3. SbS Results for DP202216 Maize (Plant ID 335728650)

This sample was negative for the DP202216 insertion as confirmed by PCR. 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 indicates endogenous genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Coverage was obtained to the same maize endogenous elements as in the [] control maize but the lack of junctions to genomic DNA indicates that the coverage is to the elements in their normal genomic context and are not related to any insertion in this plant. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the endogenous maize elements in the T-DNA region near the left of the coverage graph; however, no junctions to PHP40099 sequences were identified. The absence of any junctions to the PHP40099 sequence shows that there are no insertions or backbone sequence present in this plant.

A.



B.

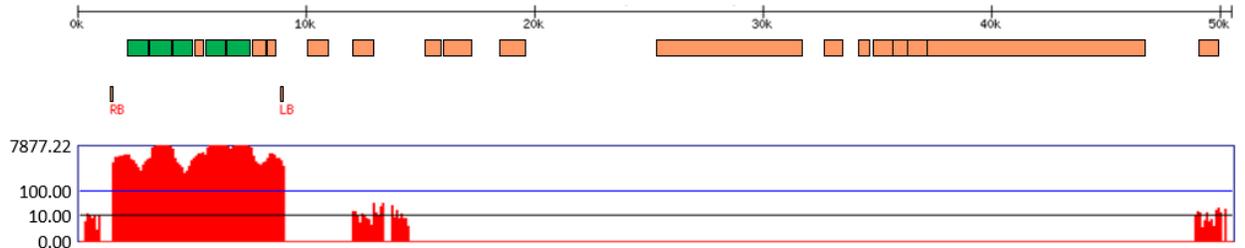
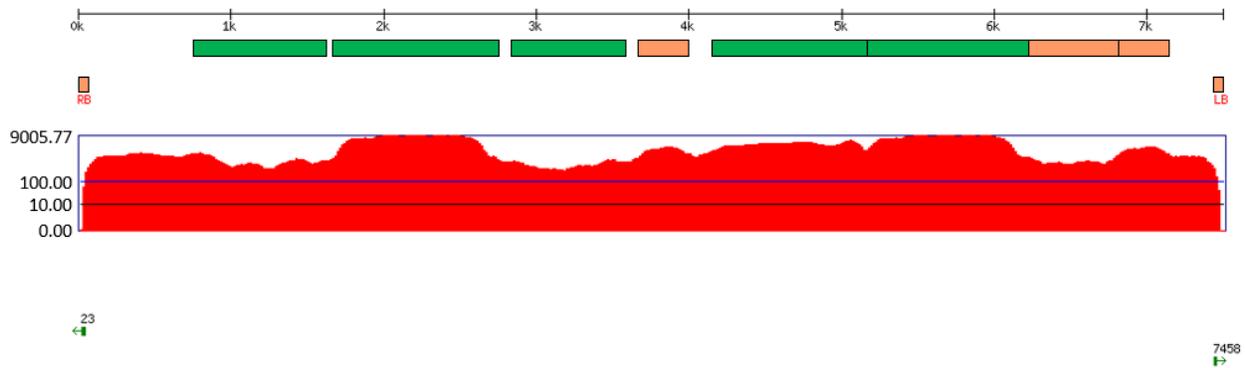


Figure A2-4. SbS Results for DP202216 Maize (Plant ID 335728651)

This sample was positive for the DP202216 insertion as confirmed by PCR. 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 indicates endogenous genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Dark green arrows in the Junctions section show 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 intact T-DNA sequence. The insertion comprises bp 23 to 7,458 of the PHP40099 T-DNA shown in Figure 6. The presence of only two junctions when aligned to the T-DNA demonstrates the presence of a single PHP40099 T-DNA in the DP202216 maize genome. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the T-DNA region near the left of the coverage graph; however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP40099 sequence shows that there are no additional insertions or backbone sequence present in DP202216 maize.

A.



B.

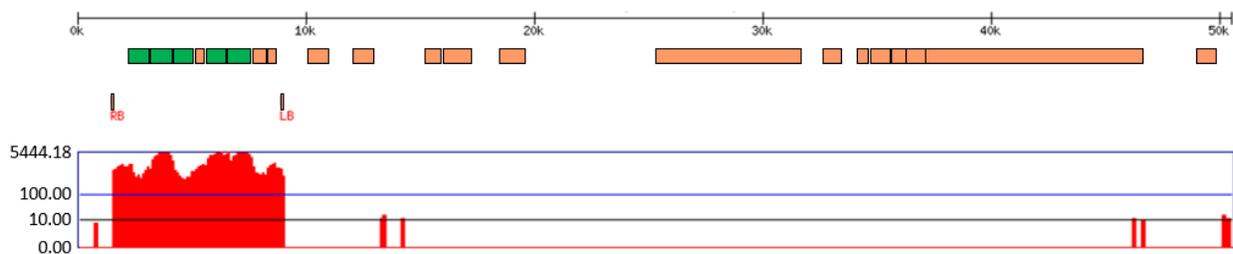
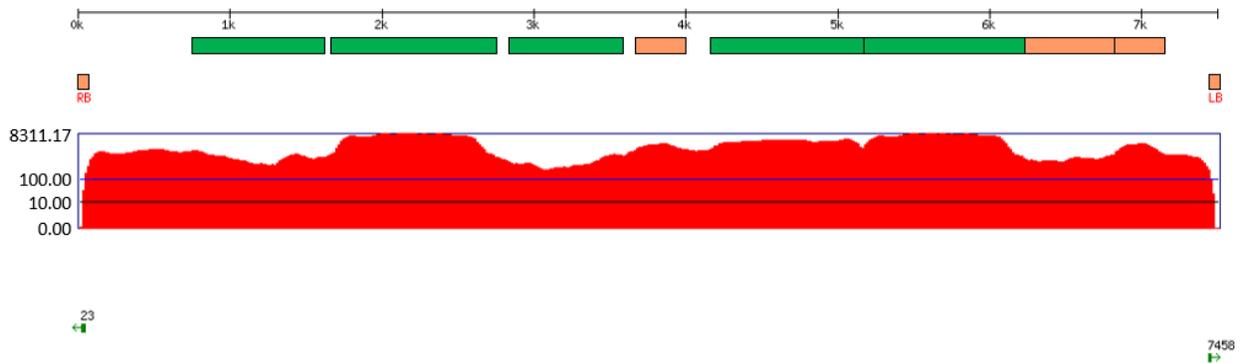


Figure A2-5. SbS Results for DP202216 Maize (Plant ID 335728652)

This sample was positive for the DP202216 insertion as confirmed by PCR. 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 indicates endogenous genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Dark green arrows in the Junctions section show 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 intact T-DNA sequence. The insertion comprises bp 23 to 7,458 of the PHP40099 T-DNA shown in Figure 6. The presence of only two junctions when aligned to the T-DNA demonstrates the presence of a single PHP40099 T-DNA in the DP202216 maize genome. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the T-DNA region near the left of the coverage graph; however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP40099 sequence shows that there are no additional insertions or backbone sequence present in DP202216 maize.

A.



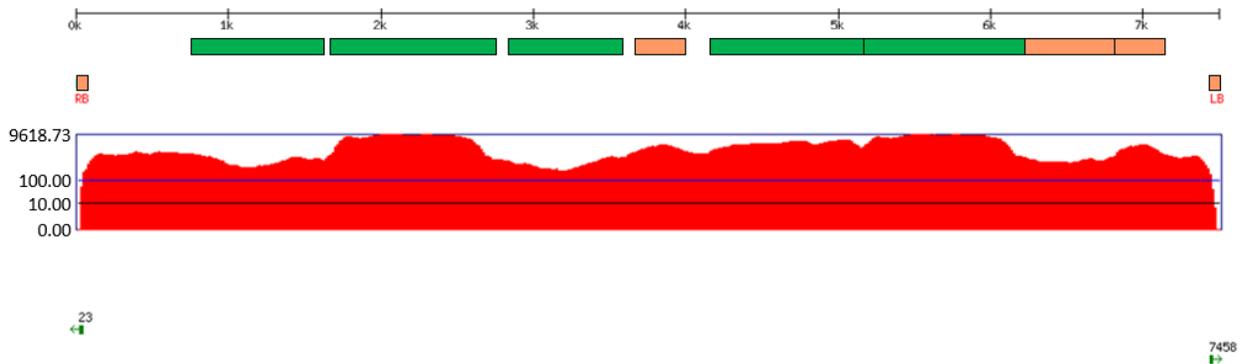
B.



Figure A2-6. SbS Results for DP202216 Maize (Plant ID 335728653)

This sample was positive for the DP202216 insertion as confirmed by PCR. 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 indicates endogenous genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Dark green arrows in the Junctions section show 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 intact T-DNA sequence. The insertion comprises bp 23 to 7,458 of the PHP40099 T-DNA shown in Figure 6. The presence of only two junctions when aligned to the T-DNA demonstrates the presence of a single PHP40099 T-DNA in the DP202216 maize genome. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the T-DNA region near the left of the coverage graph; however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP40099 sequence shows that there are no additional insertions or backbone sequence present in DP202216 maize.

A.



B.

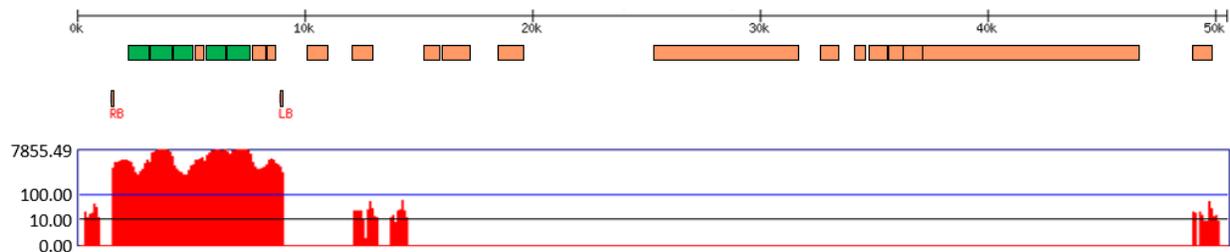


Figure A2-7. SbS Results for DP202216 Maize (Plant ID 335728654)

This sample was positive for the DP202216 insertion as confirmed by PCR. 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 indicates endogenous genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Dark green arrows in the Junctions section show 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 intact T-DNA sequence. The insertion comprises bp 23 to 7,458 of the PHP40099 T-DNA shown in Figure 6. The presence of only two junctions when aligned to the T-DNA demonstrates the presence of a single PHP40099 T-DNA in the DP202216 maize genome. **B)** SbS results aligned against the entire PHP40099 sequence (50,401 bp). Coverage was obtained for the T-DNA region near the left of the coverage graph; however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP40099 sequence shows that there are no additional insertions or backbone sequence present in DP202216 maize.

Appendix 3. Materials and Methods for Southern Blot Analysis of DP202216 Maize Test Material

Seeds from the [] generations of DP202216 maize were planted, and leaf tissue harvested from individual plants was used for genomic DNA extraction.

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Control Material

Seeds from non-GE (conventional) maize lines, [], were planted, and leaf tissue harvested from individual plants was used for genomic DNA extraction. [] generations of DP202216 maize are of the [] control maize genetic background. [] generations of DP202216 maize are of the [] control maize background.

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Reference Material

Plasmid DNA of PHP40099 (Figure 1) was used as a positive control for Southern analysis to verify probe hybridization. The *zmm28* and *mo-pat* probes used in this study were derived from plasmid PHP40099.

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were obtained from commercial vendors and were used as a reference to determine approximate molecular weights and amounts of DNA fragments. For Southern analysis, DNA Molecular Weight Marker III and VII, Digoxigenin (DIG)-labeled (Roche, Indianapolis, IN), were used as size standards for hybridizing fragments.

Sample Collection, Handling, Identification and Storage

Seed from each of the five generations of DP202216 maize and each of the control maize lines were planted in a controlled environment at Pioneer, Johnston, Iowa, USA. Fresh leaf tissue samples from test and control lines were harvested and then lyophilized. Lyophilized tissue samples were shipped to Regulatory Sciences, E.I DuPont India Pvt. Ltd, DuPont Knowledge Center, Hyderabad, at ambient temperature. Upon arrival, samples were stored frozen (< -50°C freezer unit) until processing.

Southern Analysis

Samples

Genomic DNA was isolated and analyzed from leaf tissue from five generations (one plant from each of the [] generations) of DP202216 maize and one plant from each of the [] control maize lines.

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DNA Extraction and Quantification

The leaf samples were pulverized with steel beads in tubes using a paint shaker (AGS Transact Technology Ltd., Mumbai, India). Care was taken to ensure leaf samples were ground sufficiently for DNA isolation. Genomic DNA was isolated using a high salt extraction buffer (2.0 M Sodium chloride, 100 mM Tris-Hydrochloride pH-8.0, 50 mM Sodium salt of EDTA, 3% β -mercaptoethanol (v/v) and 100 mM Sodium metabisulphite) and sequentially precipitated using potassium acetate and isopropyl alcohol. DNA was treated with Ribonuclease A, purified and precipitated using sodium acetate and chilled ethanol. Following the extraction, DNA was quantified using PicoGreen[®] reagent (Molecular Probes, Invitrogen) and visualized on a 1% agarose gel to check the quality of the isolated DNA.

Digestion of DNA and Electrophoretic Separation

Genomic DNA isolated from both test and control maize leaves was digested with the restriction enzyme *Nco* I (Thermo Fisher Scientific., Waltham, MA, USA). PHP40099 plasmid DNA was added to control plant DNA samples at a level equivalent to one plasmid copy per genomic copy and digested in the same manner. Following digestion with the restriction enzyme, the fragments produced were electrophoretically separated according to their sizes using an agarose gel and documented by photographing the gel under UV illumination (BioRad Gel doc XR⁺ System., Hercules, CA, USA).

Southern Transfer

The DNA fragments separated on the agarose gel were denatured *in situ*, transferred to a nylon membrane (GE Healthcare, LC, Buckinghamshire, UK) and fixed to the membrane by UV crosslinking (UV Stratalinker, UVP, Cambridge, UK).

Probe Labeling and Southern Blot Hybridization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probe. DNA probes specific to the *zmm28* and *mo-pat* genes (Figure 2) were labeled by incorporation of Digoxigenin (DIG) labeled nucleotide (DIG-11-dUTP) into the fragments.

Labeled probes were hybridized to the DNA on the nylon membrane for detection of the specific genomic DNA fragments. DNA Molecular Weight Marker III and VII, Digoxigenin (DIG) labeled (Roche, Indianapolis, IN, USA) were used for visualization as the fragment size standards on the blot.

Detection of Hybridized Probes

After stringent washes, DIG-labeled DNA standards and single stranded DIG-labeled probes hybridized to DNA bound to the nylon membrane were visualized using CDP (Chemiluminescent Disodium 2-chloro-5-(4-methoxyspiro(1,2-dioxetane-3,2'-(5-chlorotricyclo(3.3.1.1^{3,7})decan))-4-yl)-1-phenyl phosphate)-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche, Indianapolis, IN, USA). Blots were exposed for one or more time points to detect hybridizing fragments and to visualize molecular weight standards. Images were captured by detection with the Syngene G-Box Chemi XT16 and XX6 (Syngene, Inc., Cambridge, UK). Detected bands were documented for each probe.

Stripping of Probes and Subsequent Hybridization

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

Appendix 4. Materials and Methods for Segregation Analysis of Five Generations of DP202216 Maize

Five generations of DP202216 maize were evaluated using polymerase chain reaction (PCR) analyses and herbicide-resistance testing to confirm Mendelian inheritance of the genotype and phenotype.

Greenhouse Experimental Design

Five separate generations [] of DP202216 maize were planted and grown in a greenhouse under standard environmental conditions for maize production. Leaf samples were collected from each generation and analyzed using PCR amplification methods specific for the event DP-202216-6, *mo-pat* gene, and *zmm28* gene. After sample collection, all plants were treated with a broadcast application of glufosinate and then visually evaluated for herbicide resistance.

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Planting and Leaf Sample Collection

Maize seeds, 135-165 for each generation, were planted in separate 4-inch pots contained in flats of 15 pots each and grown in a controlled environment under conditions for producing maize plants. Thirteen to fourteen days after planting, each generation was thinned to a final population of approximately 100 plants.

When plants were at the V3 growth stage (the growth stage when the collar of the third leaf is visible) and prior to herbicide application, leaf samples were collected from each plant. Each sample consisted of three leaf punches collected into one bullet tube and placed on dry ice until transferred to a freezer (≤ -80 °C) for storage. Individual plant and corresponding leaf samples were uniquely labeled to allow a given sample to be tracked back to the originating plant.

Genotypic Analysis

Leaf samples were analyzed using an event-specific PCR assay to confirm the presence or absence of event DP-202216-6, and gene-specific PCR assays to confirm the presence or absence of the *mo-pat* and *zmm28* genes.

Phenotypic Analysis

For the [] generations, glufosinate-ammonium was applied after PCR leaf punch sample collection. At the time of herbicide application, the maize plants were at the V4 growth stage. The spray mixture consisted of Ignite 280 SL containing 24.5% glufosinate-ammonium and ammonium sulfate at a rate of approximately 3.0 lb/A (3.4 kg/ha). No other adjuvants or additives were included in the spray mixture. Ignite 280 SL was applied at a target rate of 22 fl oz/A (1.66 L/ha) with a total spray volume of approximately 33

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gal/A (312.4 L/ha) using a spray chamber to simulate a broadcast (over-the-top) application. Actual application rates were within 90-110% of the target herbicide application rate.

Five to six days after herbicide application, each plant (total of 100 plants per entry) was visually evaluated for herbicide resistance in which presence of herbicide injury corresponded to an herbicide-susceptible phenotype and absence of herbicide injury corresponded to an herbicide-resistant phenotype.

Statistical Analysis

A chi-square analysis was performed at the 0.05 significance level on the segregation results of each DP202216 maize generation to compare the observed segregation ratio to the expected segregation ratio (3:1 for [] and 1:1 for []). This analysis tested the hypothesis that the introduced traits segregated according to the Mendelian rules of inheritance. The critical value to reject the hypothesis at the 5% level is 3.84. Chi-square test was not performed for [] generations because all plants were identified as positive (*i.e.*, not segregating) as expected for a homozygous generation.

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Appendix 5. Materials and Methods for Determination of ZMM28 and PAT Protein Concentrations

Field Trial Experimental Design

The field portion of this study was conducted during the 2017 growing season at six sites in maize-growing regions of the United States (one site in each of Iowa, Indiana, Missouri, Nebraska, and Pennsylvania) and Canada (one site in Ontario). Each site included DP202216 maize and control maize. A randomized complete block design with four blocks was utilized at each site.

Sample Collection

Leaf (V6, V9, R1, R4, and R6 growth stages), pollen (R1 growth stage), root (V9, R1, R4, and R6 growth stage), forage (R4 growth stage), whole plant (V9, R1, and R6 growth stages), and grain (R6 growth stage) samples from all four blocks were collected at each site from DP202216 maize and control maize for expressed trait protein analysis. One sample per plot was collected from two self-pollinated rows 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. Control maize samples were collected prior to the collection of the corresponding DP202216 maize samples to minimize potential contamination. 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, 50-ml vial.

Pollen

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

Forage

Each forage sample was obtained by cutting the plants approximately 4-6 in. (10-15 cm) above the soil surface line. The aerial portion of 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.

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 aerial portion of 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. 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. The R6 whole plant samples included the husk and cob from the sampled plants; however, the grain was removed and used for the respective grain sample.

Grain

Each grain sample was obtained by husking and shelling the grain from one selected ear. The plants selected for grain sampling contained self-pollinated ears. For each sample, a representative sub-sample of 15 kernels was collected into an individual pre-labeled, 35-ml 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). Forage and whole plant 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.

Protein Concentration Determination

The concentrations of ZMM28 and PAT proteins were determined using quantitative enzyme-linked immunosorbent assays (ELISA) that have been internally validated to demonstrate method suitability. The ZMM28 ELISA could not be validated for grain due to matrix issues, therefore, a western blot method that was developed and internally validated was used to quantify ZMM28 protein in grain. The ZMM28 protein is expressed in both the DP202216 maize

and control maize samples, therefore, expression was measured in all tissue samples. The gene encoding PAT protein is not present in the control maize samples, and therefore, PAT protein was not measured in control tissue samples.

Protein Extraction

Processed tissue sub-samples were weighed at the following target weights: 5 mg for pollen; 10 mg for leaf; 20 mg for grain and root; and 30 mg for forage and whole plant.

Each pollen, leaf, forage, root, and whole plant sample analyzed for ZMM28 protein was extracted with 0.60 ml of chilled buffer, which was comprised of 0.25% amidosulfobetaine-14 (ASB-14) in phosphate-buffered saline containing polysorbate 20 (PBST). Each grain sample analyzed for ZMM28 protein concentration was extracted in 0.60 ml of lithium dodecyl sulfate with dithiothreitol (LDS/DTT) extraction buffer. Samples analyzed for PAT protein concentration were extracted in 0.60 ml of chilled PBST. Extracted samples were centrifuged, and then supernatants were removed and prepared for analysis.

ZMM28 Protein ELISA Method for Maize Leaf, Pollen, Forage, Root, and Whole Plant Tissues

Prior to analysis, leaf, pollen, forage, root, and whole plant samples were diluted as applicable in PBST with 0.25% ASB-14. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with a ZMM28-specific antibody. Following incubation, unbound substances were washed from the plate. A different ZMM28-specific antibody, conjugated to the enzyme horseradish peroxidase (HRP), was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound ZMM28-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.

ZMM28 Protein Western Blot Method for Maize Grain

Standard curves were prepared in a diluent of grain matrix extract and then samples and standards were heated at 95 °C for 5-6 minutes. Standards (typically analyzed in single lanes), grain samples (typically analyzed in duplicate lanes), and a protein molecular weight marker to provide visualization of migration were loaded to a NuPAGE polyacrylamide gel. Electrophoresis was conducted at a constant 200 volts (V).

Following PAGE, separated proteins were transferred from the gel to a nitrocellulose membrane using an iBlot Gel Transfer Stack. Following protein transfer, the membrane was blocked in non-fat dry milk and incubated in a 1:3000 dilution of ZMM28-specific mouse monoclonal antibody

8H10.26.16 (Pioneer Hi-Bred International, Inc.). Following primary antibody incubation, the membrane was washed to remove unbound substances and then incubated in a 1:5000 dilution secondary antibody (anti-mouse IgG horseradish peroxidase conjugate). Unbound substances were washed from the membrane prior to incubating in a chemiluminescent substrate. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

The intensity of chemiluminescent light emitted was directly related to the amount of ZMM28 protein present in the treated sample extract. Carestream imaging software was utilized for defining and analyzing luminescent intensity (regions of interest (ROIs)) from the captured image. ROI data were exported to SoftMax Pro GxP for sample interpolation. The equation for each standard curve was derived by the software, which used a quadratic fit to relate the ROI value obtained for each standard lane to the respective standard concentration (ng/ml).

PAT Protein ELISA Method for Maize Tissues

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

Calculations for Determining ZMM28 and PAT Protein Concentrations by ELISA

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

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

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

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

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

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

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

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

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

$$\text{Adjusted Concentration} = \text{Interpolated Sample Concentration} \times \text{Dilution Factor}$$

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

$$\text{Adjusted Concentration} = 3.6 \text{ ng/ml} \times 20 = 72 \text{ ng/ml}$$

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

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

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

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

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

$$\text{Reportable Assay LLOQ (ng/ml)} = (\text{lowest standard concentration} - 10\%) \times \text{minimum dilution}$$

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

$$\text{Reportable Assay LLOQ (ng/ml)} = (0.50 \text{ ng/ml} - (0.50 \times 0.10)) \times 10 = 4.5 \text{ ng/ml}$$

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

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

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 ZMM28 Protein Concentrations by Western Blot

SoftMax Pro GxP (Molecular Devices) microplate data software was used to perform the calculations required to convert the ROI intensity values obtained for grain samples to a protein concentration value.

A standard curve was included on each western blot. 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 ROI intensity value (ROI)

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

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

For example, given curve parameters of $A = 109362$, $B = 2388676$, $C = 39500$, and a sample ROI = 1399660

$$\text{Sample Concentration} = \frac{-2388676 + \sqrt{2388676^2 - 4(39500)(109362 - 1399660)}}{2(39500)} = 0.54 \text{ ng/ml}$$

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

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

For example, sample concentration = 0.54 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 20 mg

$$\text{Sample Concentration (ng protein/mg sample weight)} = 0.54 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{20 \text{ mg}} = 0.016 \text{ ng/mg}$$

The reportable assay 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 = 0.25 ng/ml and minimum dilution = 1

Reportable Assay LLOQ (ng/ml) = (0.25 ng/ml - (0.25 x 0.10)) x 1 = 0.23 ng/ml

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

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

For example, reportable assay LLOQ = 0.23 ng/ml, extraction buffer volume = 0.60 ml, and sample target weight = 20 mg

$$\text{LLOQ} = 0.23 \text{ ng/ml} \times \frac{0.60 \text{ ml}}{20 \text{ mg}} = 0.0069 \text{ ng/mg sample weight}$$

Statistical Analysis

Statistical analysis of the protein concentration results consisted of the calculations of means, ranges, and standard deviations.

Appendix 6. Methods for Protein Characterization and Equivalency Analysis

ZMM28 Western Blot Methods

Protein Sample Preparation

Leaf (V9 growth stage; Abendroth et al., 2011) and grain (R6 growth stage) samples were collected from DP202216 maize and near-isoline maize for western blot analysis. Samples were collected from plants grown, lyophilized, processed, and stored at ≤ -10 °C under study PHI-2017-005.

The lyophilized leaf and grain samples were extracted with 1X LDS/DTT (25% 4X NuPAGE lithium dodecyl sulfate (LDS) Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent with dithiothreitol (DTT) and 65% ASTM Type 1 water) and then clarified by centrifugation.

Leaf tissue samples were diluted with 1X LDS/DTT and heat treated at 90-100 °C to prepare for Polyacrylamide Gel Electrophoresis (PAGE). Samples were stored frozen at ≤ -10 °C.

Polyacrylamide Gel Electrophoresis (PAGE)

LDS/DTT treated samples stored at ≤ -10 °C were re-heated at 90-100 °C and then loaded into a 4-12% Bis-Tris gel. Prestained protein molecular weight markers (Bio-Rad 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 running buffer at a constant 200 volts (V).

Upon completion of electrophoresis, the gel was prepared for protein transfer to a membrane for western blot analysis.

Western Blot Analysis

Following PAGE, separated proteins were transferred from the gel to a nitrocellulose membrane using an iBlot Gel Transfer Stack. Following protein transfer, the membrane was blocked in non-fat dry milk and incubated in a 1:3000 dilution of ZMM28 mouse monoclonal antibody 8H10.26.16 (Pioneer Hi-Bred International, Inc.). Following primary antibody incubation, the membrane was washed to remove unbound substances and then incubated in a 1:5000 dilution of secondary antibody (anti-mouse IgG horseradish peroxidase conjugate). Unbound substances were washed from the membrane prior to incubating in a chemiluminescent substrate. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

PAT Protein Western Blot Methods

Materials

The samples analyzed were leaf tissue samples from the V9 growth stage of development (Abendroth et al., 2011), grown from the DP202216 and near-isoline control maize lines. Samples were collected from plants grown at Pioneer, lyophilized, processed, and stored at ≤ -10 °C.

Protein Sample Preparation and Extraction

The lyophilized leaf samples were weighed into 1.2-ml tubes at a target weight of 10 mg ($\pm 5\%$). Samples were extracted with 0.6 ml of phosphate-buffered saline containing polysorbate 20 (PBST) extraction buffer and then clarified by centrifugation.

Following extraction and centrifugation, each tissue extract sample and analytical protein standard was prepared for SDS-PAGE. Tissue samples were formulated using 2X NuPage lithium dodecyl sulfate (LDS) sample buffer containing NuPage reducing agent (50% 4X NuPAGE LDS Sample Buffer, 20% 10X NuPAGE Sample Reducing Agent with dithiothreitol (DTT) and 30% ASTM Type 1 water). Tissue samples were diluted with 1X LDS/DTT (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent with DTT and 65% ASTM Type 1 water) to a concentration appropriate for the sensitivity of the assay and to target the same load as the analytical protein standard. The analytical protein standard was also prepared by dilution in 1X LDS/DTT. Samples were heat treated at 90-100 °C for 5 minutes and stored frozen at ≤ -10 °C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

LDS/DTT treated samples stored at ≤ -10 °C were re-heated for 5 minutes at 90-100 °C and then loaded into a 4-12% Bis-Tris gel. Pre-stained 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 and NuPAGE Antioxidant at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gel was prepared for protein transfer to a membrane 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 5 minutes to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with a PAT monoclonal antibody 22G6 (Pioneer Hi-Bred International, Inc.) diluted 1:5,000 in PBST containing 1% w/v non-fat dry milk. Following primary antibody incubation, the membrane was washed 3 times in PBST for 5 minutes each. The membrane was incubated for 60 minutes at ambient laboratory temperature with a secondary antibody (anti-mouse IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:20,000 in PBST containing 1% non-fat dry milk. The membrane was washed 3 times with PBST for 5 minutes each. The blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

Appendix 7. Methods of Heat Lability of PAT Protein

The effect of temperature on the structure of the PAT proteins was examined (Hérouet et al., 2005). The PAT proteins were dissolved in 20 mM Tris-HCl and 5 mM ethylenediaminetetraacetic acid (EDTA) buffer at a concentration of 0.25 mg/ml in 1.5-ml microcentrifuge tubes. They were tested for stability at temperatures of 60, 75, and 90 °C for periods of 10, 30, and 60 minutes in a temperature-controlled heating block. The heat treatment was terminated by placing the sample tubes on ice, and adding 14 µl distilled water and 14 µl Laemmli buffer adjusted to pH 6.8. Two control samples of a 0-minute incubation of the proteins (kept at 4 °C) were also prepared as well as two control buffer solutions without protein, heated for 60 minutes at 60 and 90 °C, respectively. The resulting proteins were analyzed by SDS-PAGE.

Appendix 8. Methods of Acute Oral Toxicity of PAT Protein

The PAT protein (84% pure or 0.84 mg PAT/mg powder) was evaluated for acute oral toxicity (Brooks, 2000). Five male and five female CD-1 mice received 6,000 mg of test material per kg body weight. The test material was administered as a 25% weight per volume suspension in aqueous 0.5% methylcellulose. Since the volume of test material in the suspension exceeded 2 ml per 100g body weight, the test material suspension was administered as two fractional gavage doses approximately one hour apart. Each animal was weighed pre-study, the day of test material administration, and on test days 2, 8, and 15. All animals were observed daily for clinical signs for 15 days. At study termination, animals were euthanized. All animals were examined for gross pathologic changes. Means and standard deviations were calculated for body weights.

Appendix 9. Materials and Methods for Nutrient Composition

Field Trial Experimental Design

The field portion of this study was conducted during the 2017 growing season at eight sites in maize-growing regions of the United States (one site in each of Iowa, Illinois, Indiana, Missouri, Nebraska, Pennsylvania, and Texas) and Canada (one site in Ontario). Each site included DP202216 maize, control maize, and conventional reference maize lines. A randomized complete block design with four blocks was utilized at each site.

Sample Collection

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected from DP202216 maize, control maize, and conventional reference maize lines. One sample per plot was collected and all samples were collected from impartially selected, healthy, representative plants. Each sample was uniquely labeled with a sample identification number and barcode for sample tracking, and is traceable by site, entry, block, tissue, and growth stage.

Forage

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

Grain

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

Each forage and grain sample was placed in chilled storage (*e.g.*, coolers with wet ice, artificial ice, dry ice, or in a freezer), then transferred to a freezer (≤ -10 °C). Samples were shipped frozen to Pioneer Hi-Bred International, Inc., and then shipped frozen to EPL Bio Analytical Services (EPL BAS, Niantic, IL, USA) for analyses or shipped frozen directly to EPL BAS.

Nutrient Composition Analyses

Nutrient composition analyses of forage and grain samples were conducted by EPL BAS. All procedures and methods used by EPL BAS are described in Table A9-1. Nutrient composition analyses of forage and grain samples included the determination of the following analytes:

Proximates, Fiber, and Minerals Composition in Forage

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

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

Proximates and Fiber Composition in Grain

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

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

Fatty 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

- Calcium
- Copper
- Iron
- Magnesium
- Manganese
- Phosphorus
- Potassium
- Sodium
- 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

Table A9-1. Methods for Compositional Analysis of DP202216 Maize

Nutritional Analyte	Method
Moisture Forage and Grain	The analytical procedure for moisture determination was based on a method published by the Association of Official Analytical Chemists (AOAC). Samples were assayed to determine the percentage of moisture by gravimetric measurement of weight loss after drying in a forced air oven (forage) and a vacuum oven (grain).
Ash Forage and Grain	The analytical procedure for ash determination was based on a method published by the AOAC. Samples were analyzed to determine the percentage of ash by gravimetric measurement of the weight loss after ignition in a muffle furnace.
Crude Protein Forage and Grain	The analytical procedure for crude protein determination utilized an automated Kjeldahl technique based on a method provided by the manufacturer of the titrator unit (Foss-Tecator) and the AOAC. Ground samples were digested in the presence of a catalyst. The digestate was then distilled and titrated with a Foss-Tecator Kjeltac Analyzer unit.
Crude Fat Forage and Grain	The analytical procedure for crude fat determination was based on methods provided by the American Oil Chemists' Society (AOCS) and the manufacturer of the hydrolysis and extraction apparatus (Ankom Technology). Samples were hydrolyzed with 3N hydrochloric acid at 90 °C for 80 minutes for forage and 60 minutes for grain. The hydrolysates were extracted with a petroleum ether/ethyl ether/ethyl alcohol solution at 90 °C for 60 minutes. The ether extracts were evaporated and the fat residue remaining determined gravimetrically.
Carbohydrates Forage and Grain	The carbohydrate content in maize forage and grain on a dry weight basis was calculated using a formula obtained from the United States Department of Agriculture " <i>Energy Value of Foods</i> ," in which the percent dry weight of crude protein, crude fat, and ash was subtracted from 100%.
Crude Fiber Forage and Grain	The analytical procedure for crude fiber determination was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC, and the AOCS. Samples were analyzed to determine the percentage of crude fiber by digestion and solubilization of other materials present.
Neutral Detergent Fiber	The analytical procedure for neutral detergent fiber (NDF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC, and the <i>Journal of AOAC International</i> . Samples were analyzed to determine the percentage of NDF by digesting with a neutral detergent solution, sodium sulfite, and alpha amylase. The remaining residue was dried and weighed to determine the NDF content.
Acid Detergent Fiber Forage and Grain	The analytical procedure for acid detergent fiber (ADF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology) and the AOAC. Samples were analyzed to determine the percentage of ADF by digesting with an acid detergent solution and washing with reverse osmosis water. The remaining residue was dried and weighed to determine the ADF content.

Table A9-1. Methods for Compositional Analysis of DP202216 Maize (continued)

Nutritional Analyte	Method
Total Dietary Fiber	The analytical procedure for the determination of total dietary fiber in grain was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC, and the manufacturer of the protein titrator unit (Foss-Tecator). Duplicate samples were gelatinized with heat stable α -amylase, enzymatically digested with protease and amyloglucosidase to remove protein and starch, respectively, and then soluble dietary fiber precipitated with ethanol. The precipitate (residue) was quantified gravimetrically. Protein analysis was performed on one of the duplicate samples while the other duplicate sample was analyzed for ash. The weight of the protein and ash was subtracted from the weight of the residue divided by sample dry weight.
Minerals	The analytical procedure for the determination of minerals is based on methods published by the AOAC and CEM Corporation. The maize forage minerals determined were calcium and phosphorus. Additional grain minerals determined were copper, iron, magnesium, manganese, potassium, sodium, and zinc. The samples were digested in a microwave based digestion system and the digestate was diluted using deionized water. Samples were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES).
Tryptophan	The analytical procedure for tryptophan determination was based on an established lithium hydroxide hydrolysis procedure with reverse phase ultra performance liquid chromatography (UPLC) with ultraviolet (UV) detection published by the <i>Journal of Micronutrient Analysis</i> .
Cystine and Methionine	The analytical procedure for cystine and methionine determination was based on methods obtained from Waters Corporation, AOAC, and <i>Journal of Chromatography A</i> . The procedure converts cystine to cysteic acid and methionine to methionine sulfone, after acid oxidation and hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives which are then analyzed by reverse phase UPLC with UV detection.
Additional Amino Acids	Along with tryptophan, cystine, and methionine, 15 additional amino acids were determined. The analytical procedure for analysis of these amino acids was based on methods obtained from Waters Corporation and the <i>Journal of Chromatography A</i> . The procedure converts the free acids, after acid hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives, which are analyzed by reverse phase UPLC with UV detection.
Fatty Acids	The analytical procedure for determination of fatty acids was based on methods published by the AOAC and AOCS. The procedure converts the free acids, after ether extraction and base hydrolysis, to the fatty acid methyl ester (FAME) derivatives, which are analyzed by gas chromatography with flame ionization detection (GC/FID). Results are reported as percent total fatty acids but presented in the raw data as percent fresh weight.
Thiamine (Vitamin B1) and Riboflavin (Vitamin B2)	The analytical procedure for the determination of thiamine (vitamin B1) and riboflavin (vitamin B2) was based on a method published by the American Association of Cereal Chemists (AACC). The samples were extracted with 10% acetic acid/4.3% trichloroacetic acid solution. A 50-fold dilution was performed and then the samples were analyzed by reverse phase high pressure liquid chromatography (HPLC) tandem mass spectrometry (MS/MS).

Table A9-1. Methods for Compositional Analysis of DP202216 Maize (continued)

Nutritional Analyte	Method
Niacin (Vitamin B3)	The analytical procedure for the determination of niacin (vitamin B3) was based on a method published by the AACC. Niacin (vitamin B3) was extracted from the sample by adding deionized (DI) water and autoclaving. A tube array was prepared using three different dilutions of the samples. This tube array was inoculated with <i>Lactobacillus plantarum</i> and allowed to incubate for approximately 18 to 22 hours. After incubation, the bacterial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of nicotinic acid.
Pantothenic Acid (Vitamin B5)	The analytical procedure for the determination of pantothenic acid (vitamin B5) was based on a method from the AOAC. Pantothenic acid (vitamin B5) was determined using a microbiological assay. Pantothenic acid (vitamin B5) was extracted from the sample by adding an acetic acid buffer solution and autoclaving. The pH was adjusted and a tube array was prepared using three different dilutions of the samples. This tube array was inoculated with <i>Lactobacillus plantarum</i> and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of D-pantothenic acid hemicalcium salt.
Pyridoxine (Vitamin B6)	The analytical procedure for the determination of pyridoxine (vitamin B6) was based on a method from the AACC. Pyridoxine (vitamin B6) was determined using a microbiological assay. Pyridoxine (vitamin B6) was extracted from the sample by adding sulfuric acid and autoclaving. The pH was adjusted and a tube array was prepared using four different dilutions of the samples. This tube array was inoculated with <i>Saccharomyces cerevisiae</i> and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 600 nm. The absorbance readings were compared to a standard curve generated using known concentrations of pyridoxine hydrochloride.
Total Folate as Folic Acid (Vitamin B9)	The analytical procedure for determination of total folate as folic acid was based on a microbiological assay published by the AACC. Samples were hydrolyzed and digested by protease and amylase enzymes to release the folate from the grain. A conjugase enzyme was used to convert the naturally occurring folypolyglutamates. An aliquot of the extracted folates was mixed with a folate and folic acid free microbiological growth medium. The mixture was inoculated with <i>Lactobacillus casei</i> . The total folate content was determined by measuring the turbidity of the <i>Lactobacillus casei</i> growth response in the sample and comparing it to the turbidity of the growth response with folic acid standards using a spectrophotometer at 600 nm.

Table A9-1. Methods for Compositional Analysis of DP202216 Maize (continued)

Nutritional Analyte	Method
Total Tocopherols	The analytical procedure for determination of tocopherols was based on methods from the <i>Journal of the American Oil Chemists' Society</i> and <i>Analytical Sciences</i> . Alpha, beta, gamma, and delta tocopherols were extracted with hot hexane and the extracts were analyzed by normal phase UPLC with fluorescence detection.
Beta-Carotene	The analytical procedure for determination of beta-carotene was based on a method published by the AOAC. Samples were extracted using a 40:60 acetone:hexane with tert-butylhydroquinone (TBHQ) solution then analyzed by HPLC-UV.
Trypsin Inhibitor	The analytical procedure for the determination of trypsin inhibitor was based on a method published by the AOCS. Trypsin inhibitor was extracted with sodium hydroxide. Benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) was added and reacted with trypsin inhibitor. The amount of trypsin activity present in the reaction was measured using a spectrophotometer, and the amount of inhibitor was calculated based on the inhibition of trypsin activity.
Inositol and Raffinose	The analytical procedure for the determination of inositol and raffinose was based on a gas chromatography (GC) method published in the <i>Handbook of Analytical Derivatization Reactions</i> , an AACC method, and a method from the <i>Journal of Agricultural and Food Chemistry</i> . Extracted inositol and raffinose were analyzed by reverse phase HPLC with refractive index detection.
Furfural	The analytical procedure for the determination of furfural was based on methods published in the <i>Journal of Agricultural and Food Chemistry</i> . Ground maize grain was analyzed for furfural content by reverse phase HPLC with UV detection.
<i>p</i> -Coumaric and Ferulic Acid	The analytical procedure for the determination of <i>p</i> -coumaric and ferulic acids was developed based on methods published in <i>Journal of Agricultural and Food Chemistry</i> and <i>The Journal of Chemical Ecology</i> . Ground maize grain was analyzed to determine the amounts of <i>p</i> -coumaric acid and ferulic acid by separating the total content of phenolic acids using reverse phase HPLC and UV detection.
Phytic Acid	The analytical procedure for the determination of phytic acid was based on a method published by the AOAC. The samples were analyzed to determine the amount of phytic acid by extracting the phytic acid with dilute hydrochloric acid (HCl) and isolating it using an aminopropyl silica solid phase extraction column. Once isolated and eluted, the phytic acid was analyzed for elemental phosphorus by ICP-OES.

Statistical Methods

Statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC, USA) to evaluate and compare the nutrient composition of forage and grain derived from DP202216 maize and the control maize.

Processing of Data

Values Below Lower Limit of Quantification

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

Conversion of Fatty Acid Assay Values

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

For a given sample, the conversion to units of % total fatty acids 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.

One fatty acid, erucic acid (C22:1), was excluded from the conversion and from statistical analyses because all sample values in the current study and in historical conventional reference maize lies were below the LLOQ.

Calculation of Total Tocopherol

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

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

Selection of Statistical Method

For a given analyte, the number of samples below the assay LLOQ value determined how the statistical analyses were conducted. The following rules were implemented:

- If both DP202216 maize and the control maize had < 50% of samples across sites below the LLOQ, then mixed model was applied in the across-site analysis.
- If either DP202216 maize or the control maize had $\geq 50\%$ samples below the LLOQ, but not both entries had 100% of samples below the LLOQ across sites, then Fisher's exact test would be conducted. The Fisher's exact test assessed whether there was a significant difference (P-value < 0.05) in the proportion of samples below the LLOQ between these two maize lines across sites.
- If both DP202216 maize and the control maize had 100% of samples below the LLOQ, then statistical analyses were not performed.

Statistical Model for Across-Site Analysis

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

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

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

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 DP202216 maize and the control maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method (Kenward and Roger, 1997). A significant difference was identified if a P-value was < 0.05.

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

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.

Interpretations of Statistical Results

For a given analyte, when a statistically significant difference (P-value from mixed model analysis < 0.05, or Fisher's exact test P-value < 0.05) was identified in the across-site analysis, the respective range of individual values from DP202216 maize was compared to a tolerance interval. Tolerance intervals are expected to contain at least 99% of the values for corresponding analytes of the conventional maize population with a 95% confidence level (Hong et al., 2014). The tolerance intervals were derived from Pioneer's proprietary accumulated data from non-GE maize lines, which were grown in maize-growing regions in the United States, Canada, and South America between 2003 and 2015. The combined data represent 93 conventional maize lines and 88 unique environments. The selected conventional maize lines represent the non-GE maize population with a history of safe use, and the selected environments (site and year combinations) represent maize growth under a wide range of environmental conditions (*i.e.* soil texture, temperature, precipitation, and irrigation) and maize maturity group zones.

If the range of DP202216 maize contained individual values outside the tolerance interval, it was then compared to the respective literature range obtained from published literature (Codex Alimentarius Commission, 2013; Cong et al., 2015; ILSI, 2016; Lundry et al., 2013; OECD, 2002; Watson, 1982). Literature ranges compliment tolerance intervals in that they are composed of non-proprietary data from additional non-GE conventional maize lines and growing environments, which are not included in Pioneer's proprietary database.

If the range of DP202216 maize contained individual values outside the literature range, it was then compared to the respective in-study reference range comprised of all individual values across-sites from all conventional 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.

Reported Statistics

The statistical results for transformed data were back-transformed to the original data scale for reporting purposes. For each analyte, LS Means (back-transformed, if needed), ranges, and 95% confidence intervals (back-transformed, if needed) (labeled as Mean, Range, and Confidence Interval, respectively) are provided in Tables 12-19 for the across-site analysis. Both the FDR-adjusted P-values and non-adjusted P-values (labeled as Adjusted P-Value and P-Value, respectively) are provided for comparisons between DP202216 maize and the control maize. For each analyte, a tolerance interval and a literature range, if available, are provided. All analytes with sample values below the LLOQ, as well as the numbers of sample values below the LLOQ and P-values of Fisher's exact test, are provided.

Descriptive statistics (arithmetic means and ranges) are reported for analytes that were not statistically analyzed using mixed model analyses. 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 $<LLOQ$.

Appendix 10. Materials and Methods for Human Dietary Exposure

Materials

Maize grain samples were collected from field sites in the United States and were analyzed for the target protein concentrations. The mean concentrations of ZMM28 and PAT proteins expressed in DP202216 maize grain were 0.012 and 15 µg/g dry weight, respectively (Table A10-1).

Methods

The dietary exposure to ZMM28 and PAT proteins was estimated using the DEEM™ - FCID program. Annual mean and 95th percentile daily exposures were calculated for the United States population and several sub-populations for a conservative hypothetical total replacement scenario (100% DP202216 maize inclusion), assuming that all maize products consumed are derived from DP202216 maize grain.

DEEM™ - FCID categorizes maize (field corn) consumption as: flour, flour-baby food, meal, meal-baby food, bran, starch, starch-baby food, syrup, syrup-baby food, oil, and oil-baby food. For the purposes of this exposure assessment, it was assumed that corn flour, corn flour-baby food, corn meal, corn meal-baby food, and corn bran contain ZMM28 and PAT proteins at levels equivalent to the mean concentrations in grain (Table A10-1). Because the processing conditions would denature and remove virtually all proteins in corn starch, corn starch-baby food, corn syrup, corn syrup-baby food, corn oil, and corn oil-baby food (CRA, 2006a; CRA, 2006b; CRA, 2006c; Hefle and Taylor, 1999), these processed commodities were assumed to contain no ZMM28 or PAT proteins.

Dietary risk can be characterized by calculating the amount of grain that would have to be consumed to expose a person to the same level of protein used in an acute toxicity study in mice where no treatment-related adverse effects were observed over a 14-day period following oral gavage with the microbially-produced protein. The estimated amount of grain is then evaluated in terms of how feasible it would be for a person to eat that amount of grain in one day.

Results and Discussion

Mean annual (chronic) exposure was highest for the 'children ages 3-5 years' subgroup with exposures of 0.000007 and 0.008285 mg/kg body weight/day for ZMM28 and PAT proteins, respectively (Table A10-2). The highest 95th percentile per capita daily (acute) exposure for ZMM28 was in the 'children ages 1-2 years' and 'children ages 3-5 years' subgroups with exposures of 0.000027 mg/kg body weight/day; the highest 95th percentile per capita daily (acute) exposure for PAT was in the 'children ages 3-5 years' subgroup with an exposure of 0.034106 mg/kg body weight/day (Table A10-3). The highest 95th percentile users daily (acute)

exposures for ZMM28 and PAT proteins were in the ‘children ages 1-2 years’ subgroup with exposures of 0.000036 and 0.044548 mg/kg body weight/day, respectively.

The actual exposure to these proteins in the diet is expected to be even lower because (1) maize grain is highly blended, thus DP202216 grain containing ZMM28 and PAT proteins will be mixed with other grain potentially not containing these proteins and (2) reductions in protein concentrations will occur during processing to produce maize flour and other processed products.

In the case of PAT protein, 20,000 kg DP202216 maize grain would have to be eaten in one day by a 60-kg adult and 3,333 kg eaten by a 10 kg child (Table A10-1) to equal the amount consumed by mice in a 14-day acute toxicology study where no treatment-related adverse effects were observed (Brooks, 2000). Because ZMM28 is a native maize protein and has a history of safe use, no acute toxicology studies were conducted; however, it is worth noting the estimated DDEs to ZMM28 protein were approximately 650 to 1250-fold less than the corresponding values for PAT.

Table A10-1. Mean Expression Concentrations of ZMM28 and PAT Proteins in DP202216 Maize Grain and Estimated Grain Consumption Necessary to Match Acute Toxicity Test Dose

Protein	Mean Concentration ^a in DP202216 Maize Grain (mg/kg)	Acute Toxicity Test Dose (mg/kg BW/day)	DP202216 Maize Grain Consumption Necessary to Match Acute Toxicity Test Dose (kg/day) ^b	
			Adult (60 kg)	Child (10 kg)
ZMM28	0.012 ^c	NA	NA	NA
PAT	15	5000 ^d	20,000	3,333

Note: NA = not available

^a Concentrations in dry weight are from section VI-B of this petition.

^b DP202216 maize grain consumption necessary to match acute toxicology test dose where no treatment-related effects were observed (mg/kg BW/day) = acute toxicology dose * body weight (kg) / expression (µg/g)

^c 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 mean and standard deviation.

^d Brooks (2000)

**Table A10-2. DEEM™ - FCID Dietary Exposure Assessment
Mean Annual (Chronic) Results for DP202216 Maize Proteins**

Population Subgroup	Annual Mean (Chronic) Exposure (mg/kg body weight/day) ^a	
	ZMM28	PAT
U.S. Population	0.000002	0.002992
Hispanics	0.000006	0.007180
Non-Hispanic Whites	0.000002	0.002128
Non-Hispanic Blacks	0.000003	0.003126
Non-Hispanic Other	0.000002	0.002234
Nursing Infants	0.000001	0.001077
Non-nursing Infants	0.000001	0.001645
Female 13+ yrs pregnant	0.000003	0.003355
Children 1-6 yrs	0.000006	0.007963
Children 7-12 yrs	0.000005	0.005778
Male 13-19 yrs	0.000003	0.003802
Female 13-19 yrs/non-pregnant	0.000003	0.003155
Male 20+ yrs	0.000002	0.002369
Female 20+ yrs/non-pregnant	0.000001	0.001789
Seniors 55+ yrs	0.000001	0.001432
All Infants	0.000001	0.001466
Female 13-50 yrs	0.000002	0.002365
Children 1-2 yrs	0.000006	0.007397
Children 3-5 yrs	0.000007	0.008285
Children 6-12 yrs	0.000005	0.006137
Youth 13-19 yrs	0.000003	0.003482
Adults 20-49 yrs	0.000002	0.002514
Adults 50-99 yrs	0.000001	0.001506
Female 13-49 yrs	0.000002	0.002390

^a Includes all corn flour, meal and bran; assuming all derived from DP202216 maize grain with no protein degradation due to processing

Table A10-3. ZMM28 and PAT Protein Concentrations Utilized in the Dietary Exposure Assessment of DP202216 Maize

Maize Processed Fraction	DEEM™ - FCID Category	ZMM28 ^a	PAT ^a
		(mg/kg)	
Flour (fines)	Corn, field, flour	0.012	15
Flour (fines)	Corn, field, flour - baby food	0.012	15
Flaking (large) grits	Corn, field, meal	0.012	15
Flaking (large) grits	Corn, field, meal - baby food	0.012	15
Bran	Corn, field, bran	0.012	15

^a Mean ZMM28 and PAT protein concentrations are from section VI-B of this petition.

Appendix 11. Materials and Methods for Livestock Dietary Exposure

Materials

Maize grain and forage samples were collected from field sites in the United States and were analyzed for the targeted protein concentrations. The mean concentrations of ZMM28 and PAT proteins in DP202216 maize grain were 0.012 and 15 mg/kg dry weight, respectively (Table A11-1). The mean concentrations in DP202216 maize forage were 0.049 and 32 mg/kg dry weight, respectively.

Methods

Daily dietary exposure (DDE) to the ZMM28 and PAT proteins from DP202216 maize grain and forage were calculated for various livestock species using estimates of animal body weight, daily feed intake, and grain inclusion rates specific for NA (OECD, 2013; corn, field). The following conservative total replacement scenarios were utilized:

- 100% DP202216 maize grain replacement for poultry (broiler, layer, turkey), swine (breeding, finishing), cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP202216 maize forage replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb);
- 100% DP202216 maize grain and forage combination replacement for cattle (beef, dairy) and sheep (ram/ewe, lamb)

Results and Discussion

Refer to Table A11-2 for daily dietary intake of maize grain by the various livestock species. Refer to Table A11-3 for estimated DDEs to ZMM28 and PAT proteins by various livestock species consuming DP202216 maize grain.

The highest estimated DDE with 100% maize grain replacement was observed in broilers with values of 0.000818 and 1.02 mg/kg body weight/day for ZMM28 and PAT, respectively (Table A11-3). The highest estimated DDE for maize forage or maize grain+forage replacement was in dairy with DDE values from maize forage consumption of 0.00221 and 1.44 mg/kg body weight/day for ZMM28 and PAT, respectively, and DDE values from maize grain+forage consumption of 0.002450 and 1.75 mg/kg body weight/day, respectively. The estimated DDEs to ZMM28 protein were approximately 650 to 1250-fold less than the corresponding values for PAT.

In practice, the actual livestock dietary exposures to these proteins are expected to be even lower than these estimates because (1) maize grain is highly blended, thus maize sources containing ZMM28 and PAT proteins will be mixed with other maize grain sources potentially not containing these proteins, and (2) the estimates were highly conservative in their maize incorporation rates,

not accounting for typical blending with other feedstuffs for adequate nutrient levels and least-cost formulations.

The estimated DDE to the PAT protein, based on conservative assumptions (*e.g.*, all maize grain in the diet derived from DP202216 maize) was compared with the dose administered in an acute oral toxicity study in mice where no treatment-related effects were observed (5000 mg/kg body weight/day (Brooks, 2000) to determine margin of exposure (MOE). The MOEs ranged across species and total replacement scenarios from 2,862 to 46,588 (Table A10-4). Because ZMM28 is a native maize protein and has a history of safe use, no acute toxicology study was conducted.

In conclusion, exposure to ZMM28 and PAT proteins from consumption of DP202216 maize grain products is very low. There is a clear margin of safety for the PAT protein expressed in DP202216 maize as consumption will remain far below that which was consumed in the acute toxicity study where no treatment-related adverse effects were observed. ZMM28 protein is a native maize protein and as such has a history of safe use. It is also very low expressing in DP202216 maize. Therefore, consumption of DP202216 maize grain is not expected to pose a risk to humans, especially when accounting for blending and processing.

Table A11-1. Mean Concentrations of ZMM28 and PAT Proteins in DP202216 Maize Grain and Forage

Protein	Mean Concentration mg/kg Dry Weight ^a	
	DP202216 Maize Grain	DP202216 Maize Forage
ZMM28	0.012	0.049
PAT	15	32

^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 mean and standard deviation.

Table A11-2. Maize Forage/Silage and Grain Consumption by Various Livestock

Animal		Body Weight (kg) ^a	Total Daily Feed Intake (kg DM/Animal) ^a	Corn, Field – Forage/Silage Inclusion Rate ^a	Corn Forage/Silage Daily Dietary Intake (g DM Feed/kg Body Weight) ^b	Corn, Field – Grain Inclusion Rate ^a	Corn Grain Daily Dietary Intake (g DM Feed/kg Body Weight) ^b
Poultry	Broiler	2	0.16	*	*	75%	68.2
	Layer	1.9	0.12	*	*	75%	53.8
	Turkey	8	0.5	*	*	75%	53.3
Swine	Breeding	270	2	*	*	85%	7.2
	Finishing	100	3.1	*	*	85%	29.9
Cattle	Beef	500	9.1	15%	6.8	80%	16.5
	Dairy	600	24	45%	45.0	45%	20.5
Sheep	Ram/Ewe	85	2	45%	26.5	45%	12.0
	Lamb	40	1.5	45%	42.2	45%	19.2

Notes: * Not used or is minor feedstuff (<5% of diet); DM = dry matter

^a CA/US-specific values for animal body weight, feed consumption and feedstuff inclusion rates were taken from OECD (2013). The inclusion rates represent the proportion of daily ration on an as-fed basis.

^b Maize forage/silage or grain daily dietary intake (g DM feed/kg body weight) = (Total daily feed intake (kg DM/animal) / Body weight (kg)) x (1000 g/1 kg) x (forage/silage or grain inclusion rate (%)/dry matter of forage/silage or grain (%)). (maize forage/silage DM=40%; maize grain DM=88% (OECD, 2013))

Table A11-3. Estimated Daily Dietary Exposures (DDEs) to ZMM28 and PAT Proteins by Various Livestock Consuming DP202216 Maize Forage/Silage and/or Grain

Animal		DDE ^a (mg/kg Body Weight)					
		ZMM28 (0.049 mg/kg DM forage/silage; 0.012 mg/kg DM grain) ^b			PAT (32 mg/kg DM forage/silage; 15 mg/kg DM grain) ^b		
		DP202216 Forage/Silage	DP202216 Grain	DP202216 Forage/Silage +Grain	DP202216 Forage/Silage	DP202216 Grain	DP202216 Forage/Silage +Grain
Poultry	Broiler	NA	0.000818	NA	NA	1.02	NA
	Layer		0.000646			0.807	
	Turkey		0.000639			0.799	
Swine	Breeding	NA	0.0000859	NA	NA	0.107	NA
	Finishing		0.000359			0.449	
Cattle	Beef	0.000334	0.000199	0.000533	0.218	0.248	0.467
	Dairy	0.00221	0.000245	0.00245	1.44	0.307	1.75
Sheep	Ram/Ewe	0.00130	0.000144	0.00144	0.847	0.180	1.03
	Lamb	0.00207	0.000230	0.00230	1.35	0.288	1.64

Notes: DM = dry matter; NA = Not Applicable; forage/silage is not used or is minor feedstuff (<5% of diet)

^a DDE = Daily Dietary Exposure; calculated by multiplying ZMM28 or PAT concentrations (mg/kg DM), respectively, by the daily dietary intake (g DM feed/kg body weight) for maize grain (Table 2) for each animal species, then multiplying by 1 kg/1000g.

^b Mean concentration of the proteins in forage/silage or grain derived from DP202216 maize presented in section VI-B of this petition.

Table A11-4. Margin of Exposures (MOEs) Using the Administered Doses of PAT Protein from Acute Oral Toxicity Study in Mice

Animal		MOE ^a		
		PAT (Dose of 5000 mg/kg Body Weight) ^a		
		DP202216 Forage/Silage	DP202216 Grain	DP202216 Forage/Silage +Grain
Poultry	Broiler	NA	4,889	NA
	Layer		6,193	
	Turkey		6,258	
Swine	Breeding		46,588	
	Finishing		11,132	
Cattle	Beef	22,894	20,147	10,716
	Dairy	3,472	16,296	2,862
Sheep	Ram/Ewe	5,903	27,704	4,866
	Lamb	3,704	17,383	3,053

Note: NA = Not Applicable; forage/silage is not used or is minor feedstuff (<5% of diet)

^a MOE = Margin of Exposure; calculated by dividing the PAT (Brooks, 2000) dose of 5000 mg/kg body weight determined from acute oral toxicity testing in mice by the respective DDE (Table A9-3). Values were calculated using raw (i.e., unrounded) data to provide a greater level of accuracy, and as such may not be directly calculated from the rounded data presented in Table A11-3

Appendix 12. Methods for Agronomic Performance Evaluation

Field Trial Experimental Design

The field portion of this study was conducted during the 2017 growing season at 12 sites in maize-growing regions of the United States (three sites in Iowa, two sites in Illinois, and one site in each of Indiana, Kansas, Missouri, Nebraska, Pennsylvania, and Texas) and Canada (one site in Ontario). A map of the approximate location of each site is provided in section VIII-B above. A randomized complete block design with four blocks was utilized at each site. Each block included DP202216 maize, non-genetically engineered (non-GE) near-isoline control maize (referred to as control maize), and four of the following non-GE conventional maize lines: 34N84, 35F38, 35P12, P0506, P0589, P0760, P0965, P0987, P0993, XL5140, XL5513, XL5828, XL5840, BK5883, XL5939, and BK6076 (referred to as reference maize).

Each block contained DP202216 maize, 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 m) in length and 30 in. (75 cm) in width. Each block was separated by an alley of at least 3 ft (1 m) in width, and each plot was bordered on either side by one row of conventional maize.

Maintenance products were uniformly applied, as needed, at each site in order to minimize weed, insect, and disease pressure. Glufosinate-ammonium, nicosulfuron, diflufenzopyr, and dicamba herbicides were not used post emergence as maintenance pesticides in this study. To control experimental bias in this study, the following procedures were utilized: non-systematic selection of trial and plot areas within each site, randomization of maize entries within each block, and uniform maintenance across blocks in each field site.

A nicosulfuron, diflufenzopyr, and dicamba herbicide treatment was applied broadcast as a tank mix to all control maize and reference maize plots, and to one of the two plots of DP202216 maize per block. Details regarding herbicide treatments are provided in Table A12-1. A visual evaluation of the plants was completed 10-17 days after each treatment to confirm no unexpected herbicide injury was observed.



Reference	Site Code	Site Location
1	RG005IA1	Richland, Iowa USA
2	RG005IA5	Atlantic, Iowa USA
3	RG005IA7	Johnston, Iowa USA
4	RG005IL5	Stewardson, Illinois USA
5	RG005IL7	Carlyle, Illinois USA
6	RG005IN2	Sheridan, Indiana USA
7	RG005KS1	Larned, Kansas USA
8	RG005MO5	Fisk, Missouri USA
9	RG005NE5	York, Nebraska USA
10	RG005ON3	Guelph, Ontario, Canada
11	RG005PA1	Germansville, Pennsylvania USA
12	RG005TX7	Groom, Texas USA

Figure A12-1. Approximate Locations and Field Site Information

Table A12-1 Herbicide Treatments

Growth Stage	Herbicide Product Name	Active Ingredient	Product Formulation	Target Rate	Non-Ionic Surfactant	Ammonium Sulfate
V4	Accent	Nicosulfuron	75% ai by weight	2/3 oz/A (46.7 g/ha)	0.25% v/v	3.0 lb/A (3.4 kg/ha)
	Status	Diflufenopyr	0.16 lb ae/lb (0.16 kg ae/kg)	10 oz/A (0.70 kg/ha)		
		Dicamba	0.40 lb ae/lb (0.40 kg ae/kg)			

Note: acid equivalent (ae), acre (A), active ingredient (ai), grams (g), hectare (ha), kilograms (kg), pound (lb), and volume per volume (v/v). Accent and Status were applied broadcast as a tank mix to all plots of control maize and reference maize, and to one of the two plots of DP202216 maize. Growth stage descriptions (Abendroth et al., 2011).

Table A12-2 Maize Growth Stage Descriptions

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.

Agronomic Characteristic Evaluation

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.

Days to Flowering

The date when approximately 50% of plants in Rows 1-4 had begun shedding pollen was recorded. These dates were used in subsequent statistical analysis to calculate days to flowering.

Plant Height

Plant height was measured in centimeters from the soil surface to the collar of the flag leaf (base of the tassel) for five individual plants in Rows 1-4 at the R4 growth stage; with the exception of site RG005ON3 where some plots were near or at the R5 growth stages.

Lodging

Lodging was evaluated at the R6 growth stage in Rows 1-4. 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 the majority of the plants in Rows 1 and 2 first reached physiological maturity was recorded.

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 grains was assessed at four time points by recording the percentage of grains with collapsed walls and yellow color (Luna et al., 2001).

Number of Kernel Rows per Ear

The total number of kernel rows per ear from each of five primary ears collected from Rows 1 and/or 2 was recorded.

Number of Kernels per Row

The total number of kernels in each of 4 rows on each of five primary ears was recorded. The same five ears were selected for evaluation of kernel rows per ear and kernels per row.

Number of Kernels per Ear

The total number of kernels per ear for five primary ears was calculated by multiplying the number of kernel rows per ear by the average number of kernels per row.

Harvest Grain Moisture

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

Yield

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

100-Kernel Weight

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

The following exceptions occurred during agronomic characteristics data collection. Plant height and lodging were collected from Rows 3 and 4 at site RG005IA1 as irrigation was only applied to Rows 3-6. Stalk lodging was not evaluated for all plots at site RG005IN2 on the same day; therefore, these data will be included in the study records but are not considered appropriate for statistical analysis. A planting error at RG005TX7 impacted the following data for one plot each of BK6076 maize, 34N84 maize, and control maize: early stand count and final stand count from Rows 1 and 2 were reported; days to flowering, pollen viability, plant height, lodging, yield, harvest grain moisture, and 100-kernel weight were evaluated from Rows 1 and 2; days to maturity, kernel rows per ear, kernels per row, and kernels per ear were evaluated from Row 3.

Statistical Methods

Statistical analyses were conducted to evaluate and compare agronomic characteristics of DP202216 maize and the control maize.

Processing of Data

Early Stand Count and Final Stand Count

For early stand count and final stand count data, the recorded count value was divided by count area to calculate the number of plants per m².

Days to Flowering and Days to Maturity

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

Plant Height

For plant height, 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. Stalk lodging was not

evaluated for all plots at site RG005IN2 on the same day; therefore, these data were not considered appropriate for statistical analysis.

Yield

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

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

$$\text{Grain dry weight (lb)} = \text{Grain fresh weight (lb)} \times (1 - \% \text{ actual moisture})$$

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

$$\text{Grain weight at 15.5\% moisture (lb)} = \text{Grain dry weight (lb)} / (1 - 15.5\% \text{ moisture})$$

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

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

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

$$\text{plot area (ft}^2) = \text{row length (ft)} \times \text{row width (ft)} \times \text{number of rows.}$$

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):

$$100\text{-kernel dry weight (g)} = 100\text{-kernel fresh weight (g)} \times (1 - \% \text{ actual moisture})$$

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

$$100\text{-kernel weight at 15.5\% moisture (g)} = 100\text{-kernel dry weight (g)} / (1 - 15.5\% \text{ moisture})$$

Kernel Rows per Ear (5 plants), Kernels per Row (4 rows x 5 plants), and Kernels per Ear (5 plants)

Recorded values of kernels per rows from 4 rows were used to calculate the plant average.

kernels per ear = kernel rows per ear × the average number of kernels per rows

Recorded or calculated values for five individual plants were used to calculate the plot average.

Selection of Statistical Method

The following rules were implemented for each agronomic characteristic:

- If < 50% of sites had uniform data values for either DP202216 maize or the control maize, and < 50% of all data across sites for each entry were at a uniform value, then an across-site mixed model analysis would be conducted.
- If ≥ 50% of sites had uniform data values for either DP202216 maize or the control maize, and ≥ 50% of sites had uniform data values across both maize lines, then statistical analyses would not be performed.
- If the criteria described above were not met, then an across-site analysis using the generalized Cochran-Mantel-Haenszel (CMH) test would be conducted.

Statistical Model for Across-Site Analysis

Mixed Model Analysis

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

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

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

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 DP202216 maize 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 .

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.

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 including simulation were 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 DP202216 maize was compared to the in-study reference range comprised of all individual values across-sites from all non-GE conventional 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.

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 13. Field Insect and Disease Observations and Methods

Experiment A - 2017 Field Trial Biotic and Abiotic Stressor Measurement

The field portion of this study was conducted during the 2017 growing season at 12 sites in maize-growing regions of the United States (three sites in Iowa, two sites in Illinois, and one site in each of Indiana, Kansas, Missouri, Nebraska, Pennsylvania, and Texas) and Canada (one site in Ontario). 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 A13-1 to A13-12.

Table A13-1. Biotic and Abiotic Observations Across Blocks at Site RG0051A1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	35P12 Maize	P0506 Maize	P0589 Maize	XL5513 Maize
R1-R2 Growth Stage							
Insect Damage	FB	None	None-Slight	None-Slight	None-Slight	None-Slight	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-Slight	None	None-Slight	None	None	None
	RSC	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	SS	None-Slight	None-Slight	None-Slight	None-Slight	None	None-Slight
	WD	None-Slight	None-Slight	None	None	Slight	Slight
R3-R6 Growth Stage							
Insect Damage	CEW	None	None	None-Slight	None	None	None
	ECB	None	None	None	None	None-Slight	None
	GH	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	GLS	None-Slight	None-Slight	None-Slight	None	None-Slight	None-Slight
	NLB	Slight	Slight	Slight	Slight	Slight	Slight
	RSC	Slight	Slight-Moderate	Slight	Slight	Slight-Moderate	Slight-Moderate
Abiotic Stressor	MPI	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
V2-V5 Growth Stage							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	FB	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	CS	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	MPI	Slight	Slight	Slight	Slight	Slight	Slight
	WD	Slight	Slight	Slight	Slight	Slight	Slight
	WL	Slight	Slight	Slight	Slight	Slight	Slight
V7-V9 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	BB	None	None-Slight	None-Slight	None-Slight	None-Slight	None
	ECB	None	None	None	None-Slight	None-Slight	None-Slight
Pathogen Stressor	CS	None	None-Slight	None-Slight	None	None-Slight	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

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), black cutworms (BCW), 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 blight (NLB), northern leaf spot (NLS), common maize rust (RSC), and stalk rot (SR). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), sun scald (SS), waterlogging (WL), wind damage (WD), and maintenance and non-target pesticide injury (MPI).

Table A13-2. Biotic and Abiotic Observations Across Blocks at Site RG005IA5

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	35F38 Maize	35P12 Maize	XL5140 Maize	XL5513 Maize
R1-R2 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	JP	None	None	None	None	None	None
	RW	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None	None	None	None	None	None
	RSC	Slight	Slight	Slight-Moderate	Slight-Moderate	Slight	Slight-Moderate
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	AP	Slight	Slight	Slight-Moderate	Slight-Moderate	Slight	Slight
	ECB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight-Moderate	Moderate	Slight-Moderate	Slight	Slight-Moderate
	RSC	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate
	SMT	None-Slight	None-Slight	None	None	None	None
Abiotic Stressor	HL	Slight	Slight	Slight	Slight	Slight	Slight
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
V2-V5 Growth Stage							
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
	ES	None	None	None	None	None	None
	GW	None	None	None	None	None	None
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	HS	Slight	Slight	Slight	Slight	Slight	Slight
	WD	None-Slight	None-Slight	None-Slight	None-Slight	None	None-Slight
V7-V9 Growth Stage							
Insect Damage	FB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
	JP	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	HL	None	None	None	None	None	None
	WD	None	None	None	None	None	None

Note: A rating of “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (e.g., minor feeding or minor lesions); “moderate” indicates intermediate between slight and severe; and “severe” indicates symptoms damaging to plant development (e.g., stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), billbugs weevils (BB), European corn borer (ECB), black cutworms (BCW), flea beetles (FB), grasshoppers (GH), Japanese beetles (JP), and adult rootworms (RW). Pathogen stressors consisted of anthracnose (AN), eyespot (ES), Goss’s bacterial wilt (GW), grey leaf spot (GLS), northern leaf blight (NLB), common maize rust (RSC), and common smut (SMT). Abiotic stressors consisted of drought (DR), hail (HL), heat stress (HS), waterlogging (WL), and wind damage (WD).

Table A13-3. Biotic and Abiotic Observations Across Blocks at Site RG0051A7

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	35F38 Maize	35P12 Maize	P0589 Maize	XL5140 Maize
R1-R2 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	GC	None-Slight	None-Slight	None-Slight	None	None-Slight	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-Slight	None	None-Slight	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	CEW	None	None	None	None	None	None-Slight
	ECB	None	None	None	None	None	None
	GH	None-Slight	None-Slight	None-Slight	None	None-Slight	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	RSC	Slight	Slight	Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate
	SMT	None-Slight	None-Slight	None-Slight	None-Slight	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HL	None	None	None	None	None	None
	WD	None	None-Slight	None	None-Slight	None-Slight	None-Slight
V2-V5 Growth Stage							
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 Stage							
Insect Damage	AP	None	None	None	None	None	None
	CEW	None	None	None	None	None	None
	JP	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	DR	None	None	None	None	None	None
	HL	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
	WD	Slight	Slight	Slight	Slight	Slight	Slight

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

Table A13-4. Biotic and Abiotic Observations Across Blocks at Site RG0051L5

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	34N84 Maize	P0965 Maize	P0987 Maize	BK5883 Maize
R1-R2 Growth Stage							
Insect Damage	AM	None	None-Slight	None	None	None-Slight	None
	AP	None	None	None	None	None	None
	JP	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
	RSS	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	DR	Slight	Slight	Slight	Slight	Slight	Slight
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	CEW	None	None-Slight	None	None-Slight	None-Slight	None-Slight
	ECB	None	None	None	None	None	None
	GH	None	None	None	None	None	None
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
	RSS	Slight	Slight	None-Slight	Slight	Slight	None-Slight
Abiotic Stressor	DR	None-Slight	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
V2-V5 Growth Stage							
Insect Damage	AM	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	FB	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	CS	None	None	None	None	None	None
	MDMV	None	None	None	None	None	None
	SW	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	ND	None	None	None	None	None	None
	SCP	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
	WL	None	None	None	None	None	None
V7-V9 Growth Stage							
Insect Damage	AM	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	JP	None	None	None	None	None	None
Pathogen Stressor	BSR	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None

Note: A rating of “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); “moderate” indicates intermediate between slight and severe; and “severe” indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), corn earworm (CEW), European corn borer (ECB), black cutworms (BCW), flea beetles (FB), grasshoppers (GH), and Japanese beetles (JP). Pathogen stressors consisted of bacterial stalk rot of maize (BSR), corn stunt (CS), grey leaf spot (GLS), maize rough dwarf virus (MRDV), common maize rust (RSC), southern maize rust (RSS), and Stewart’s wilt (SW). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), soil compaction (SCP), waterlogging (WL), and wind damage (WD).

Table A13-5. Biotic and Abiotic Observations Across Blocks at Site RG0051L7

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	P0987 Maize	P0993 Maize	BK5883 Maize	XL5939 Maize
R1-R2 Growth Stage							
Insect Damage	FAM	None	None	None-Slight	None-Slight	None-Slight	None-Slight
	FB	Slight	Slight	Slight	Slight	Slight	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	None-Slight	None-Slight	None	None-Slight	None
	SCP	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	CEW	None-Slight	None-Slight	Slight	None-Slight	None-Slight	None-Slight
	GH	None-Slight	Slight	Slight	None-Slight	None-Slight	None-Slight
	RW	None-Slight	None-Slight	Slight	None-Slight	None-Slight	Slight
Pathogen Stressor	AN	None	None	None	None	None	None
	ER	None-Slight	None-Slight	Slight	None-Slight	None-Slight	None-Slight
	SMT	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	SCP	None	None	None	None	None	None
V2-V5 Growth Stage							
Insect Damage	BCW	None	None	None	None	None	None
	FAM	None	None	None	None	None	None
	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 Stage							
Insect Damage	FAM	None	None	None	None	None-Slight	None
	FB	Slight	Slight	Slight	Slight	Slight	Slight
	JP	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
Pathogen Stressor	GLS	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
	SW	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	SCP	None	None	None	None	None	None
	SS	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 fall army worms (FAM), corn earworm (CEW), black cutworms (BCW), flea beetles (FB), grasshoppers (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), common smut (SMT), and Stewart’s wilt (SW). Abiotic stressors consisted of drought (DR), heat stress (HS), nutrient deficiency (ND), soil compaction (SCP), sun scald (SS), waterlogging (WL), and wind damage (WD).

Table A13-6. Biotic and Abiotic Observations Across Blocks at Site RG0051N2

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	P0760 Maize	P0993 Maize	XL5840 Maize	BK5883 Maize
R1-R2 Growth Stage							
Insect Damage	AP	Slight	Slight	None-Slight	None	None	None-Slight
	ECB	--	--	--	--	--	Slight ^a
	FAW	None	None	None	None	None	None
Pathogen Stressor	RW	None	None	None	None	None	None ^b
	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None	None	None	None	None	None
Abiotic Stressor	RSC	Slight-Moderate	Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight
	DR	None	None	None	None	None	None
	MPI	None	None	None	None	None	None
	WD	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	AP	None-Slight	None-Slight	None	None	None	None
	CEW	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
Pathogen Stressor	ECB	None	None	None	None	None	None
	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	None	None	None	None-Slight	None	None
Abiotic Stressor	RSC	Slight	Slight-Moderate	Slight	Slight-Moderate	Slight	Slight
	DR	None-Slight	None	None	None	None	None
	MPI	None	None	None	None	None	None
	ND	Slight-Moderate	Slight	Slight-Moderate	None-Moderate	None-Moderate	None-Moderate
V2-V5 Growth Stage							
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	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
Abiotic Stressor	SR	None	None	None	None	None	None
	MPI	None	None	None	None	None	None
	WD	Slight	Slight	Slight	Slight	Slight	Slight
	WL	None	None	None	None	None	None
V7-V9 Growth Stage							
Insect Damage	ECB	None	None	None	None	None	None-Slight
	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	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	MPI	None	None	None	None	None	None
	WD	None	None	None-Slight	None-Slight	None-Slight	None
	WL	None	None	None	None	None	None

Note: A rating of “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); “moderate” indicates intermediate between slight and severe; and “severe” indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), fall army worms (FAW), corn earworm (CEW), European corn borer (ECB), black cutworms (BCW), Japanese beetles (JP), adult rootworms (RW), western bean cutworm (WBC), and stink bugs (SB). 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).

^a For BK5883 maize evaluations for ECB were taken from one plot only. ECB observations were not recorded for the rest of the plots.

^b For BK5883 maize, evaluation for RW were taken from three plots only.

Table A13-7. Biotic and Abiotic Observations Across Blocks at Site RG005KS1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	P0965 Maize	P0993 Maize	XL5828 Maize	BK6076 Maize
R1-R2 Growth Stage							
Insect Damage	CEW	None	None	None	None	None	None
	FB	None-Slight	None	Slight	None-Slight	None-Slight	None
	RW	None	None	None	None	None	None
Pathogen Stressor	GLS	None-Slight	None-Slight	Slight	None	None-Slight	None-Slight
	NLS	None	None	None	None	None	None
	SMT	None	None	None	None	None	None
Abiotic Stressor	HS	None	None	None	None	None	None
	SS	None	None	None	None	None	None
	WD	None-Slight	None	None-Slight	None-Slight	None	None-Slight
R3-R6 Growth Stage							
Insect Damage	CEW	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate	Slight-Moderate
	SB	Moderate	Moderate	Moderate	Moderate	Slight-Moderate	Moderate
	WBC	None	None-Slight	None	None	None	None
Pathogen Stressor	AN	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None
	GLS	None	None	None	None	None	None
	SMT	None-Slight	None-Slight	None-Slight	None-Slight	Slight	None-Slight
Abiotic Stressor	DR	None	None	None	None	None	None
	MT	None	None	None	None	None	None
	SCP	None	None	None	None	None	None
V2-V5 Growth Stage							
Insect Damage	BCW	None	None	None	None	None	None
	FB	None	None	None	None	None	None
	SB	None-Slight	None-Slight	None	None-Slight	None-Slight	None
Pathogen Stressor	GW	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	NLS	None	None	None	None	None	None
Abiotic Stressor	CD	None	None	None	None	None	None
	WD	None-Slight	None-Slight	Slight	None-Slight	None-Slight	None-Slight
	WL	None	None	None	None	None	None
V7-V9 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	GH	None	None-Slight	None	None	None-Slight	None
Pathogen Stressor	NLB	None	None	None	None	None	None
	NLS	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None	None-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 aphids (AP), corn earworm (CEW), black cutworms (BCW), western bean cutworms (WBC), flea beetles (FB), grasshoppers (GH), adult rootworms (RW), sap beetles (SB), and stink bugs (SB). Pathogen stressors consisted of anthracnose (AN), Goss’s bacterial wilt (GW), grey leaf spot (GLS), northern leaf blight (NLB), northern leaf spot (NLS), common maize rust (RSC), and common smut (SMT). Abiotic stressors consisted of cold stress (CD), drought (DR), heat stress (HS), mineral toxicity (MT), soil compaction (SCP), sun scald (SS), waterlogging (WL), and wind damage (WD).

Table A13-8. Biotic and Abiotic Observations Across Blocks at Site RG005M05

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	34N84 Maize	XL5828 Maize	XL5840 Maize	XL5939 Maize
R1-R2 Growth Stage							
Insect Damage	AM	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
	CEW	None	None	None	None	None	None
	JP	None	None	None	None	None	None
Pathogen Stressor	CT	None	None	None	None	None	None
	NLS	None	None	None	None	None	None
	RSC	Slight	Slight	Slight	Slight	Slight	Slight
Abiotic Stressor	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	FAM	None	None	None	None-Slight	None-Slight	None-Slight
Pathogen Stressor	GLS	None	None	None	None	None	None
	RSC	Slight	Slight	Slight-Moderate	Slight	Slight-Moderate	Slight
	SMT	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
V2-V5 Growth Stage							
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	AN	None	None	None	None	None	None
	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
Abiotic Stressor	MPI	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None-Slight	Slight	None-Slight	None-Slight
V7-V9 Growth Stage							
Insect Damage	AM	None-Slight	None	None-Slight	None	None-Slight	None-Slight
	GH	None	None	None	None	None	None
	JP	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	RSC	None	None	None	None	None	None
Abiotic Stressor	HS	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None	None	None	None	None	None

Note: A rating of “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); “moderate” indicates intermediate between slight and severe; and “severe” indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), fall army worms (FAM), corn earworm (CEW), European corn borer (ECB), grasshoppers (GH), thrips (TH), and Japanese beetles (JP). Pathogen stressors consisted of anthracnose (AN), crazy top (CT), eyespot (ES), grey leaf spot (GLS), northern leaf spot (NLS), common maize rust (RSC), and common smut (SMT). Abiotic stressors consisted of heat stress (HS), nutrient deficiency (ND), waterlogging (WL), wind damage (WD), and maintenance and non-target pesticide injury (MPI).

Table A13-9. Biotic and Abiotic Observations Across Blocks at Site RG005NE5

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	P0760 Maize	P0987 Maize	XL5840 Maize	XL5939 Maize
R1-R2 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
	RW	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	GW	None	None	None	None	None	None
Abiotic Stressor	HL	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	RW	None-Slight	None-Slight	None-Slight	None	None-Slight	None
	WBC	None	None	None	None-Slight	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	GW	None	None	None	None	None	None
	SMT	None	None-Slight	None	None	None-Slight	None-Slight
Abiotic Stressor	HL	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
V2-V5 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None-Slight	None
	GW	None	None	None	None	None	None
Abiotic Stressor	HL	Slight	Slight	Slight	Slight	Slight	Slight
	WD	None-Slight	None-Slight	Slight	None-Slight	None	None
	WL	None	None	None	None	None	None
V7-V9 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
	ECB	None	None	None	None	None	None
Pathogen Stressor	ES	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	GW	None	None	None	None	None	None
Abiotic Stressor	HL	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), black cutworms (BCW), western bean cutworms (WBC), and adult rootworms (RW). Pathogen stressors consisted of eyespot (ES), Goss’s bacterial wilt (GW), grey leaf spot (GLS), and common smut (SMT). Abiotic stressors consisted of hail (HL), waterlogging (WL), and wind damage (WD).

Table A13-10. Biotic and Abiotic Observations Across Blocks at Site RG005ON3

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	35F38 Maize	P0506 Maize	P0589 Maize	XL5140 Maize
R1-R2 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	BB	None-Slight	None-Slight	None	None-Slight	None-Slight	None-Slight
	JP	None	None	None	None	None	None
Pathogen Stressor	NLB	None-Slight	None-Slight	None-Moderate	None-Slight	None-Slight	None-Slight
	RSC	Slight	Slight	Slight	None-Slight	None-Slight	None-Slight
	SMT	None	None	None	None	None	None
Abiotic Stressor	HL	None	None	None	None	None	None
	ND	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
R3-R6 Growth Stage							
Insect Damage	CEW	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
	ECB	None	None	None	None	None	None
	SM	None	None	None	None	None	None
Pathogen Stressor	NLB	None-Slight	None-Moderate	Slight	None-Slight	None-Slight	None
	RSC	Slight	Slight-Moderate	Moderate	Slight	Slight	Slight
	SMT	None	None	None	None	None	None
Abiotic Stressor	FR	None	None	None	None	None	None
	HL	None	None	None	None	None	None
	WD	None	None	None	None	None	None
V2-V5 Growth Stage							
Insect Damage	AM	None	None	None	None	None	None
	FB	None	None	None	None	None	None
	SLG	None-Slight	Slight	None-Slight	None-Slight	None-Slight	Slight-Moderate
Pathogen Stressor	NLB	None	None	None	None	None	None
	SR	None	None	None	None	None	None
	SW	None	None	None	None	None	None
Abiotic Stressor	ND	None	None	None-Slight	None	None-Slight	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
V7-V9 Growth Stage							
Insect Damage	BB	Slight	Slight-Moderate	None-Slight	Slight	None-Slight	Slight
	JP	None	None	None	None	None	None
	SLG	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
Pathogen Stressor	ES	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
	RSC	None-Slight	Slight	None-Slight	None	None-Slight	None
Abiotic Stressor	HL	None	None	None	None	None	None
	ND	None-Slight	None-Slight	None-Slight	None	None	None-Slight
	WD	None	None	None	None	None	None

Note: A rating of “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); “moderate” indicates intermediate between slight and severe; and “severe” indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), billbugs (BB), corn earworm (CEW), European corn borer (ECB), flea beetles (FB), Japanese beetles (JP), slug (SLG), and spider mites (SM). Pathogen stressors consisted of eyespot (ES), northern leaf blight (NLB), common maize rust (RSC), common smut (SMT), stalk rot (SR), and Stewart’s wilt (SW). Abiotic stressors consisted of frost (FR), hail (HL), nutrient deficiency (ND), waterlogging (WL), and wind damage (WD).

Table A13-11. Biotic and Abiotic Observations Across Blocks at Site RG005PA1

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	P0506 Maize	P0760 Maize	XL5140 Maize	XL5513 Maize
R1-R2 Growth Stage							
Insect Damage	ECB	None	None-Slight	None	None	None	None
	GH	Slight	Slight	Slight	Slight	Slight	Slight
	JP	Slight	Slight	Slight	Slight	Slight	Slight
Pathogen Stressor	GLS	Slight	Slight	Slight	Slight	Slight	Slight
	NLB	Slight	Slight	Slight	Slight	Slight	Slight
	SMT	None	None	None	None	None	None
Abiotic Stressor	SS	None	None	None	None	None	None
	WD	None	None	None	None	None	None
	WL	None	None	None	None	None	None
R3-R6 Growth Stage							
Insect Damage	AP	None-Slight	None	None-Moderate	None-Severe	None	None-Moderate
	CEW	None-Slight	None-Slight	None-Slight	None-Slight	None	None-Slight
	GH	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	Moderate	Moderate-Severe	Moderate	Moderate-Severe	Slight-Moderate	Moderate
	SMT	None	None	None	None	None	None
Abiotic Stressor	HL	None	None	None	None	None	None
	WD	None-Slight	None-Slight	None-Slight	None-Slight	None-Slight	None
	WL	None	None-Slight	None	None-Slight	None	None-Slight
V2-V5 Growth Stage							
Insect Damage	BCW	Slight	Slight	Slight	Slight	Slight	Slight
	FB	None	None	None	None	None	None
	SB	None	None	None	None	None	None
Pathogen Stressor	AN	None	None	None	None	None	None
	GLS	None	None	None	None	None	None
	NLB	None	None	None	None	None	None
Abiotic Stressor	SCP	None	None	None	None	None	None
	SCR	None	None	None	None	None	None
	WD	None	None	None	None	None	None
V7-V9 Growth Stage							
Insect Damage	GH	None	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	DM	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
	SCP	None	None-Slight	None	None-Slight	None	None
	SS	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), corn earworm (CEW), European corn borer (ECB), black cutworms (BCW), flea beetles (FB), grasshoppers (GH), Japanese beetles (JP), and sap beetles (SB). Pathogen stressors consisted of anthracnose (AN), downy mildew (DM), grey leaf spot (GLS), northern leaf blight (NLB), common maize rust (RSC), and common smut (SMT). Abiotic stressors consisted of hail (HL), soil compaction (SCP), soil crusting (SCR), sun scald (SS), waterlogging (WL), and wind damage (WD).

Table A13-12. Biotic and Abiotic Observations Across Blocks at Site RG005TX7

Observation Type	Stressor	Stressor Rating by Maize Line					
		DP202216 Maize	Control Maize	34N84 Maize	P0965 Maize	XL5828 Maize	BK6076 Maize
R1-R2 Growth Stage							
Insect Damage	AM	None-Slight	None-Slight	None-Slight	None-Slight	Slight-Moderate	None-Slight
	AP	None	None	None	None	None	None
	SM	None	None	None	None	None	None
Pathogen Stressor	RSS	None	None	None	None	None	None
	SMT	None	None	None	None	None	None
	SR	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None-Slight
R3-R6 Growth Stage							
Insect Damage	AP	None	None	None	None	None	None
	FAM	None-Slight	None-Slight	None-Slight	Slight	Slight-Moderate	Slight
	SM	None	None	None	None	None	None
Pathogen Stressor	GLS	None	None	None	None	None	None
	SMT	None	None-Slight	Slight	None-Slight	None	None-Slight
	SR	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None-Slight
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None
V2-V5 Growth Stage							
Insect Damage	AM	None	None	None	None	None	None
	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
Pathogen Stressor	CS	None	None	None	None	None	None
	MDMV	None	None	None	None	None	None
	WSMV	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	SCR	None	None	None	None	None	None
V7-V9 Growth Stage							
Insect Damage	AM	None-Slight	None-Slight	None-Slight	None-Slight	None-Moderate	None-Slight
	AP	None	None	None	None	None	None
	BCW	None	None	None	None	None	None
Pathogen Stressor	CS	None	None	None	None	None	None
	MDMV	None	None	None	None	None	None
	WSMV	None	None	None	None	None	None
Abiotic Stressor	DR	None	None	None	None	None	None
	HS	None	None	None	None	None	None
	WD	None	None	None	None	None	None

Note: A rating of “none” indicates no damage/symptoms observed; “slight” indicates symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); “moderate” indicates intermediate between slight and severe; and “severe” indicates symptoms damaging to plant development (*e.g.*, stunting or death). Insect stressors consisted of armyworms (AM), aphids (AP), fall army worms (FAM), black cutworms (BCW), and spider mites (SM). Pathogen stressors consisted of corn stunt (CS), grey leaf spot (GLS), maize dwarf mosaic virus (MDMV), southern maize rust (RSS), common smut (SMT), stalk rot (SR), and wheat streak mosaic virus (WSMV). Abiotic stressors consisted of drought (DR), heat stress (HS), soil crusting (SCR), and wind damage (WD).

Experiment B – 2009-2017 Field Insect and Disease Observations

DP202216 Maize has been field tested in the United States and Puerto Rico since 2009, 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 DP202216 maize as compared to the control line (near-isoline, non-GE maize lines) were recorded by experienced plant breeders and field staff at least every four weeks. The plant breeders and field staff were familiar with plant pathology and entomology and recorded the severity of any insect or disease in the field. These observations provide a means to determine if DP202216 maize will respond differently from conventional maize lines to insects or diseases in the environment.

A summary of the naturally-occurring insects present in the field observations and any unexpected differences seen between DP202216 maize and control lines is presented in Table A13-13. A summary of diseases present in the field observations is presented in Table A13-14.

The following scale was used to evaluate DP202216 maize and control lines (Tables A13-13 and A13-14 Range of Severity in DP202216 Maize):

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

Abiotic stressor field observations were recorded at all United States and Puerto Rico locations and are presented in Table A13-15.

In every case, DP202216 maize did not exhibit any unexpected responses to naturally-occurring insects, diseases or abiotic stressors as compared to the control line.

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?		
2009	09-033-112n	HI	Kauai	Chinese rose beetle (<i>Adoretus sinicus</i>)	mild	no		
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no		
				Lacewings (<i>Neuroptera</i>)	mild	no		
				Spider mites (<i>Tetranychidae</i>)	mild	no		
				Flea beetle (<i>Chaetocnema pulicaria</i>)	mild	no		
				Corn earworm (<i>Helicoverpa zea</i>)	mild to moderate	no		
	09-264-101n	PR	Salinas	Fall armyworm (<i>Spodoptera frugiperda</i>)	mild	no		
				Aphids (<i>Aphididae</i>)	moderate	no		
				Fall armyworm (<i>Spodoptera frugiperda</i>)	mild to moderate	no		
				Aphids (<i>Aphididae</i>)	mild to moderate	no		
				Corn earworm (<i>Helicoverpa zea</i>)	mild to moderate	no		
				Corn planthoppers (<i>Delphacidae</i>)	mild to moderate	no		
2010	09-264-101n	HI	Kauai	Corn earworm (<i>Helicoverpa zea</i>)	mild	no		
				Lacewings (<i>Neuroptera</i>)	mild	no		
				Spider mites (<i>Tetranychidae</i>)	mild	no		
				Thrips (<i>Frankliniella</i> spp.)	mild	no		
		PR	Salinas	Fall armyworm (<i>Spodoptera frugiperda</i>)	moderate	no		
				Aphids (<i>Aphididae</i>)	moderate	no		
				Spider mites (<i>Tetranychidae</i>)	moderate	no		
				Corn earworm (<i>Helicoverpa zea</i>)	moderate	no		
				Corn silk fly (<i>Euxesta stigmatias</i>)	moderate	no		
				Corn sap beetle (<i>Carpophilus</i> spp.)	moderate	no		
				HI	Kauai	Leafhopper (<i>Cicadellidae</i>)	mild	no
						Lacewings (<i>Neuroptera</i>)	mild	no
	Lady beetles (<i>Coccinellidae</i>)	mild	no					
	Beet armyworm (<i>Spodoptera exigua</i>)	mild	no					
	Corn earworm (<i>Helicoverpa zea</i>)	mild	no					
	Spider mites (<i>Tetranychidae</i>)	mild	no					
	Corn earworm (<i>Helicoverpa zea</i>)	moderate	no					
	Fall armyworm (<i>Spodoptera frugiperda</i>)	moderate	no					
	10-052-101n	PR	Santa Isabel	Aphids (<i>Aphididae</i>)	moderate	no		
				Corn planthoppers (<i>Delphacidae</i>)	moderate	no		
				IA	Polk	Corn earworm (<i>Helicoverpa zea</i>)	mild	no
						Corn earworm (<i>Helicoverpa zea</i>)	mild	no
						Lady beetles (<i>Coccinellidae</i>)	mild	no
				10-284-101n	HI	Kauai	Lacewings (<i>Neuroptera</i>)	mild
Leafhopper (<i>Cicadellidae</i>)	mild	no						
Spider mites (<i>Tetranychidae</i>)	mild	no						
Thrips (<i>Frankliniella</i> spp.)	mild	no						

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2011	11-040-122n	IA	Polk	Japanese beetle (<i>Popillia japonica</i>)	mild	no
		HI	Kauai	Leafhopper (Cicadellidae)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Stink bug (Pentatomidae)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Lacewings (Neuroptera)	mild	no
				Aphids (Aphididae)	mild	no
				Fall armyworm (<i>Spodoptera frugiperda</i>)	mild	no
	Corn earworm (<i>Helicoverpa zea</i>)	mild	no			
	11-288-101n	HI	Kauai	Aphids (Aphididae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Lacewings (Neuroptera)	mild	no
		Spider mites (Tetranychidae)	mild	no		
		PR	Ponce	Fall armyworm (<i>Spodoptera frugiperda</i>)	mild	no
				Thrips (<i>Frankliniella</i> spp.)	mild	no
				Corn planthoppers (Delphacidae)	mild	no
Salinas			Leafhopper (Cicadellidae)	mild	no	
	Spider mites (Tetranychidae)		mild	no		
Aphids (Aphididae)	mild	no				
Fall armyworm (<i>Spodoptera frugiperda</i>)	severe	no				
Corn earworm (<i>Helicoverpa zea</i>)	severe	no				
Spider mites (Tetranychidae)	mild to severe	no				
Corn silk fly (<i>Euxesta stigmatias</i>)	mild	no				

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?	
2012	12-011-102n	HI	Kauai	Aphids (Aphididae)	mild	no	
				Leafhopper (Cicadellidae)	mild	no	
				Spider mites (Tetranychidae)	mild	no	
	12-202-101n	HI	Kauai	Aphids (Aphididae)	mild	no	
				Leafhopper (Cicadellidae)	mild	no	
				Spider mites (Tetranychidae)	mild	no	
		PR	Salinas	Fall armyworm (Spodoptera frugiperda)	mild to moderate	no	
				Aphids (Aphididae)	mild to moderate	no	
				Corn planthoppers (Delphacidae)	mild to moderate	no	
				Corn silk fly (Euxesta stigmatias)	mild to moderate	no	
				Corn earworm (Helicoverpa zea)	mild	no	
Spider mites (Tetranychidae)	mild to moderate	no					
Grasshoppers (Orthoptera)	mild to moderate	no					
2013	12-202-101n	HI	Kauai	Aphids (Aphididae)	mild	no	
				Leafhopper (Cicadellidae)	mild	no	
				Spider mites (Tetranychidae)	mild	no	
	13-009-102n	HI	Kauai	Corn earworm (Helicoverpa zea)	mild	no	
				Aphids (Aphididae)	mild	no	
				Leafhopper (Cicadellidae)	mild	no	
		PR	Salinas	Spider mites (Tetranychidae)	mild	no	
				Fall armyworm (Spodoptera frugiperda)	mild to moderate	no	
				Grasshoppers (Orthoptera)	mild	no	
				Lacewings (Neuroptera)	mild	no	
				Braconid Wasps (Cheloninae)	mild	no	
				Corn planthoppers (Delphacidae)	mild	no	
				Aphids (Aphididae)	mild	no	
				Corn silk fly (Euxesta stigmatias)	mild to moderate	no	
				Corn earworm (Helicoverpa zea)	mild to moderate	no	
		Corn sap beetle (Carpophilus spp.)	mild to moderate	no			
		13-294-102n	HI	Kauai	Leafhopper (Cicadellidae)	mild	no
					Corn earworm (Helicoverpa zea)	mild	no
	Lady beetles (Coccinellidae)				mild	no	
	PR		Salinas	Spider mites (Tetranychidae)	mild	no	
				Fall armyworm (Spodoptera frugiperda)	mild to moderate	no	
Grasshoppers (Orthoptera)				mild	no		
Corn planthoppers (Delphacidae)				mild to moderate	no		
Aphids (Aphididae)				mild to moderate	no		
Corn silk fly (Euxesta stigmatias)	mild	no					
Spider mites (Tetranychidae)	mild to moderate	no					
Thrips (Frankliniella spp.)	mild	no					

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2014	14-002-104n	AR	Clay	Grasshoppers (Orthoptera)	mild	no
				Japanese beetle (<i>Popillia japonica</i>)	mild	no
				Leafhopper (Cicadellidae)	mild	no
		CA	Yolo	Grasshoppers (Orthoptera)	mild to moderate	no
				Japanese beetle (<i>Popillia japonica</i>)	mild	no
				Spider mites (Tetranychidae)	mild to severe	no
				Fall armyworm (<i>Spodoptera frugiperda</i>)	moderate	no
				Leafhopper (Cicadellidae)	mild to moderate	no
		HI	Kauai	Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Spider mites (Tetranychidae)	mild	no
		IL	Fulton	Grasshoppers (Orthoptera)	moderate	no
				European corn borer (<i>Ostrinia nubilalis</i>)	moderate	no
				Japanese beetle (<i>Popillia japonica</i>)	mild	no
				Corn rootworm (<i>Diabrotica</i> spp.)	mild	no
				Corn blotch leafminers (<i>Agromyza parvicornis</i>)	moderate	no
			Mason	European corn borer (<i>Ostrinia nubilalis</i>)	mild	no
				Corn rootworm (<i>Diabrotica</i> spp.)	mild	no
				Aphids (Aphididae)	mild	no
				Japanese beetle (<i>Popillia japonica</i>)	mild	no
			McDonough	Corn rootworm (<i>Diabrotica</i> spp.)	mild	no
Grasshoppers (Orthoptera)	mild	no				
IN	Tipton	Corn rootworm (<i>Diabrotica</i> spp.)	mild	no		

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2014	14-002-104n	IA	Jasper	Bean leaf beetles (<i>Cerotoma trifurcata</i>)	mild	no
				Corn rootworm (<i>Diabrotica</i> spp.)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Lady beetles (Coccinellidae)	mild	no
			European corn borer (<i>Ostrinia nubilalis</i>)	mild	no	
			Polk	Japanese beetle (<i>Popillia japonica</i>)	mild	no
				Corn rootworm (<i>Diabrotica</i> spp.)	mild	no
			Linn	European corn borer (<i>Ostrinia nubilalis</i>)	mild to moderate	no
		Corn earworm (<i>Helicoverpa zea</i>)		mild	no	
		Louisa	European corn borer (<i>Ostrinia nubilalis</i>)	mild	no	
			Corn earworm (<i>Helicoverpa zea</i>)	mild	no	
		NE	York	Corn rootworm (<i>Diabrotica</i> spp.)	mild	no
				Stink bug (Pentatomidae)	mild	no
		TN	Obion	Grasshoppers (Orthoptera)	mild	no
	Corn planthoppers (Delphacidae)			mild	no	
	Corn earworm (<i>Helicoverpa zea</i>)			mild	no	
	Southwestern corn borer (<i>Diatraea grandiosella</i>)			mild	no	
	Fall armyworm (<i>Spodoptera frugiperda</i>)			mild	no	
	Leafhopper (Cicadellidae)			mild	no	
	14-300-106n	HI	Kauai	Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Aphids (Aphididae)	mild to moderate	no
		PR	Salinas	Fall armyworm (<i>Spodoptera frugiperda</i>)	mild to moderate	no
				Grasshoppers (Orthoptera)	mild	no
				Corn planthoppers (Delphacidae)	mild	no
Thrips (<i>Frankliniella</i> spp.)				mild	no	
Corn silk fly (<i>Euxesta stigmatias</i>)				moderate	no	
Corn sap beetle (<i>Carpophilus</i> spp.)				moderate	no	
Spider mites (Tetranychidae)				moderate	no	
Lacewings (Neuroptera)				mild	no	
Corn earworm (<i>Helicoverpa zea</i>)	mild to moderate	no				

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?	
2015	15-012-101n	CA	Yolo	Fall armyworm (<i>Spodoptera frugiperda</i>)	mild	no	
				Mites (Acari)	moderate	no	
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no	
		HI	Kauai	Leafhopper (Cicadellidae)	mild	no	
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no	
				Lacewings (Neuroptera)	mild	no	
				Spider mites (Tetranychidae)	mild	no	
				Bureau	Japanese beetle (<i>Popillia japonica</i>)	mild	no
					Grasshoppers (Orthoptera)	mild	no
		IL	Champaign	Corn rootworm (<i>Diabrotica</i> spp.)	mild	no	
				European corn borer (<i>Ostrinia nubilalis</i>)	mild	no	
				Fall armyworm (<i>Spodoptera frugiperda</i>)	mild	no	
			Mason	Grasshoppers (Orthoptera)	mild	no	
				Corn rootworm (<i>Diabrotica</i> spp.)	mild	no	
				Japanese beetle (<i>Popillia japonica</i>)	mild	no	
		McDonough	European corn borer (<i>Ostrinia nubilalis</i>)	mild	no		
			Corn rootworm (<i>Diabrotica</i> spp.)	mild	no		
			Japanese beetle (<i>Popillia japonica</i>)	mild	no		
			Aphids (Aphididae)	mild	no		
		IA	Dallas	Grasshoppers (Orthoptera)	mild	no	
				Grasshoppers (Orthoptera)	mild	no	
				Lady beetles (Coccinellidae)	mild to moderate	no	
				European corn borer (<i>Ostrinia nubilalis</i>)	mild	no	
Aphids (Aphididae)	mild			no			
Corn rootworm (<i>Diabrotica</i> spp.)	mild			no			
Polk	Japanese beetle (<i>Popillia japonica</i>)		mild	no			
	Corn rootworm (<i>Diabrotica</i> spp.)		mild	no			
	Grasshoppers (Orthoptera)		mild	no			
	Bean leaf beetles (<i>Cerotoma trifurcata</i>)		mild	no			

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2015	15-012-101n	KS	Finney	Spider mites (Tetranychidae)	mild to moderate	no
				Corn rootworm (Diabrotica spp.)	mild	no
		NE	York	Corn rootworm (Diabrotica spp.)	mild	no
		PR	Ponce	Aphids (Aphididae)	moderate	no
				Corn earworm (Helicoverpa zea)	moderate	no
				Corn silk fly (Euxesta stigmatias)	mild to moderate	no
				Fall armyworm (Spodoptera frugiperda)	moderate	no
				Thrips (Frankliniella spp.)	moderate	no
				Lacewings (Neuroptera)	mild to moderate	no
				Spider mites (Tetranychidae)	moderate	no
			Corn planthoppers (Delphacidae)	moderate	no	
			Salinas	Aphids (Aphididae)	mild to moderate	no
				Armyworms (Spodoptera spp.)	mild to moderate	no
		Corn earworm (Helicoverpa zea)		mild to moderate	no	
		TX	Hale	Grasshoppers (Orthoptera)	mild	no
				Spider mites (Tetranychidae)	mild to moderate	no
		WI	Rock	Corn planthoppers (Delphacidae)	mild	no
			Corn sap beetle (Carpophilus spp.)	mild	no	
	15-300-101n	HI	Kauai	Spider mites (Tetranychidae)	moderate	no
				Japanese beetle (Popillia japonica)	mild	no
				Corn rootworm (Diabrotica spp.)	mild	no
				Chinese rose beetle (Adoretus sinicus)	mild	no
				Mites (Acari)	mild	no
PR		Salinas	Grasshoppers (Orthoptera)	mild	no	
			Leafhopper (Cicadellidae)	mild	no	
			Corn earworm (Helicoverpa zea)	mild	no	
			Aphids (Aphididae)	mild	no	
			Armyworms (Spodoptera spp.)	mild	no	
		Corn earworm (Helicoverpa zea)	mild	no		
		Corn planthoppers (Delphacidae)	mild	no		
		Two-spotted spider mite (Tetranychus urticae)	mild	no		

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2016	16-015-103n	CA	Yolo	Armyworms (<i>Spodoptera</i> spp.)	moderate	no
				Mites (<i>Acari</i>)	moderate to severe	no
				Corn earworm (<i>Helicoverpa zea</i>)	moderate	no
		HI	Kauai	Armyworms (<i>Spodoptera</i> spp.)	mild	no
				Leafhopper (<i>Cicadellidae</i>)	mild	no
				Chinese rose beetle (<i>Adoretus sinicus</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Mites (<i>Acari</i>)	mild	no
				European corn borer (<i>Ostrinia nubilalis</i>)	mild	no
		IL	Bureau	Japanese beetle (<i>Popillia japonica</i>)	mild	no
				Leafhopper (<i>Cicadellidae</i>)	mild	no
				Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no
				Armyworms (<i>Spodoptera</i> spp.)	mild	no
			Champaign	Corn sap beetle (<i>Carpophilus</i> spp.)	mild	no
				Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no
				Aphids (<i>Aphididae</i>)	mild	no
				Japanese beetle (<i>Popillia japonica</i>)	mild	no
			Mason	Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	moderate	no
				Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no
				Japanese beetle (<i>Popillia japonica</i>)	mild	no
McDonough	Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no			
	Grasshoppers (<i>Orthoptera</i>)	mild	no			
IA	Louisa	European corn borer (<i>Ostrinia nubilalis</i>)	mild	no		
		Grasshoppers (<i>Orthoptera</i>)	mild	no		
	Polk	Japanese beetle (<i>Popillia japonica</i>)	mild	no		

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2016	16-015-103n	KS	Finney	Banks grass mite (<i>Oligonychus pratensis</i>)	mild to moderate	no
				Two-spotted spider mite (<i>Tetranychus urticae</i>)	mild to moderate	no
				Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild to moderate	no
				Corn leafminer (<i>Agromyza</i> spp.)	mild	no
				Spotted cucumber beetle (<i>Diabrotica undecimpunctata</i>)	mild	no
				Corn silk fly (<i>Euxesta stigmatias</i>)	mild	no
			European corn borer (<i>Ostrinia nubilalis</i>)	mild	no	
			Hodgeman	Spotted cucumber beetle (<i>Diabrotica undecimpunctata</i>)	mild	no
				Banks grass mite (<i>Oligonychus pratensis</i>)	mild to moderate	no
		Two-spotted spider mite (<i>Tetranychus urticae</i>)		mild to moderate	no	
		NE	York	Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no
				Aphids (<i>Aphididae</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Two-spotted spider mite (<i>Tetranychus urticae</i>)	moderate	no
				Stink bug (<i>Pentatomidae</i>)	mild	no
				Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no
		SD	Brookings	Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no
				Mites (<i>Acari</i>)	mild	no
		TX	Hale	Corn rootworm (<i>Diabrotica</i> spp.)	mild	no
Corn earworm (<i>Helicoverpa zea</i>)	mild			no		
WI	Rock	Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no		
		Grasshoppers (<i>Orthoptera</i>)	mild	no		

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2016	16-291-102n	HI	Kauai	Leafhopper (Cicadellidae)	mild	no
				Mites (Acari)	mild	no
				Chinese rose beetle (<i>Adoretus sinicus</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
		PR	Salinas	Armyworms (<i>Spodoptera</i> spp.)	moderate to severe	no
				Leafhopper (Cicadellidae)	moderate to severe	no
				Aphids (Aphididae)	moderate	no
				Spider mites (Tetranychidae)	mild	no
				Corn silk fly (<i>Euxesta stigmatias</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	moderate	no
Corn sap beetle (<i>Carpophilus</i> spp.)	moderate	no				
2017	16-291-102n	PR	Guayama	Armyworms (<i>Spodoptera</i> spp.)	mild to moderate	no
				Leafhopper (Cicadellidae)	mild to moderate	no
				Aphids (Aphididae)	mild	no
				Corn silk fly (<i>Euxesta stigmatias</i>)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Thrips (<i>Frankliniella</i> spp.)	mild	no
			Salinas	Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Armyworms (<i>Spodoptera</i> spp.)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Corn silk fly (<i>Euxesta stigmatias</i>)	mild	no
		PR	Salinas	Spider mites (Tetranychidae)	mild	no
				Thrips (<i>Frankliniella</i> spp.)	moderate	no
				Corn sap beetle (<i>Carpophilus</i> spp.)	mild	no
				Leafhopper (Cicadellidae)	mild	no

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?			
2017	17-017-102n	CA	Yolo	Mites (Acari)	mild	no			
		HI	Kauai	Mites (Acari)	mild	no			
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no			
		IL	McDonough	Chinese rose beetle (<i>Adoretus sinicus</i>)	mild	no			
				Japanese beetle (<i>Popillia japonica</i>)	mild	no			
				Grasshoppers (Orthoptera)	mild	no			
				Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no			
				Corn flea beetle (<i>Chaetochema pulicaria</i>)	mild	no			
				Grasshoppers (Orthoptera)	mild	no			
			Champaign	Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no			
				Bureau	Japanese beetle (<i>Popillia japonica</i>)	mild	no		
					Aphids (Aphididae)	mild	no		
					Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no		
					IA	Linn	Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no
							Aphids (Aphididae)	mild	no
		Stink bug (Pentatomidae)	mild				no		
		Polk	Japanese beetle (<i>Popillia japonica</i>)	mild	no				
			Aphids (Aphididae)	mild	no				
			Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no				
		KS	Finney	Spotted cucumber beetle (<i>Diabrotica undecimpunctata</i>)	mild	no			
				Banks grass mite (<i>Oligonychus pratensis</i>)	mild to moderate	no			
				Two-spotted spider mite (<i>Tetranychus urticae</i>)	mild to moderate	no			
				Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild to moderate	no			
				Stink bug (Pentatomidae)	mild	no			
				Grasshoppers (Orthoptera)	mild	no			
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no			
			Hodgeman	Spotted cucumber beetle (<i>Diabrotica undecimpunctata</i>)	mild	no			
				Banks grass mite (<i>Oligonychus pratensis</i>)	mild to moderate	no			
Two-spotted spider mite (<i>Tetranychus urticae</i>)	mild to moderate			no					
Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild to moderate			no					
Stink bug (Pentatomidae)	mild			no					
Grasshoppers (Orthoptera)	mild			no					
Corn earworm (<i>Helicoverpa zea</i>)	mild	no							

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-017-102n	NE	York	Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no
		PR	Salinas	Armyworms (<i>Spodoptera</i> spp.)	mild to moderate	no
				Leafhopper (<i>Cicadellidae</i>)	mild to moderate	no
				Spider mites (<i>Tetranychidae</i>)	mild	no
				Corn silk fly (<i>Euxesta stigmatias</i>)	mild to moderate	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Corn sap beetle (<i>Carpophilus</i> spp.)	mild	no
				Thrips (<i>Frankliniella</i> spp.)	mild	no
				Grasshoppers (<i>Orthoptera</i>)	mild to moderate	no
				Aphids (<i>Aphididae</i>)	mild	no
				Whiteflies (<i>Aleyrodidae</i>)	mild	no
		Aibonito	Armyworms (<i>Spodoptera</i> spp.)	mild	no	
			Corn planthoppers (<i>Delphacidae</i>)	mild to moderate	no	
			Corn silk fly (<i>Euxesta stigmatias</i>)	mild	no	
			Two-spotted spider mite (<i>Tetranychus urticae</i>)	mild	no	
			Thrips (<i>Frankliniella</i> spp.)	mild	no	
			Leafhopper (<i>Cicadellidae</i>)	mild	no	
		TN	Obion	Spotted cucumber beetle (<i>Diabrotica undecimpunctata</i>)	mild	no
				Stink bug (<i>Pentatomidae</i>)	mild	no
	Armyworms (<i>Spodoptera</i> spp.)			mild to moderate	no	
	Corn earworm (<i>Helicoverpa zea</i>)			mild to moderate	no	
	TX	Hale	Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no	
Northern corn rootworm (<i>Diabrotica barberi</i>)			mild	no		
17-019-101n	IL	Shelby	Japanese beetle (<i>Popillia japonica</i>)	mild	no	
			Grasshoppers (<i>Orthoptera</i>)	mild	no	
			Corn earworm (<i>Helicoverpa zea</i>)	mild	no	

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-019-101n	IN	Boone	Armyworms (<i>Spodoptera</i> spp.)	mild	no
				Aphids (<i>Aphididae</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
		IA	Audubon	Black cutworm (<i>Agrotis ipsilon</i>)	mild	no
				Aphids (<i>Aphididae</i>)	mild	no
				Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no
				Western corn rootworm (<i>Diabrotica virgifera virgifera</i>)	mild	no
				Grasshoppers (<i>Orthoptera</i>)	mild	no
				Grasshoppers (<i>Orthoptera</i>)	mild	no
		KS	Pawnee	Stink bug (<i>Pentatomidae</i>)	mild	no
				Corn flea beetle (<i>Chaetochema pulicaria</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				European corn borer (<i>Ostrinia nubilalis</i>)	mild	no
		MO	Butler	Japanese beetle (<i>Popillia japonica</i>)	mild	no
				Spotted cucumber beetle (<i>Diabrotica undecimpunctata</i>)	mild	no
				Stink bug (<i>Pentatomidae</i>)	mild	no
				Tarnished Plantbug (<i>Lygus lineolaris</i>)	mild	no
		NE	York	Northern corn rootworm (<i>Diabrotica barberi</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Grasshoppers (<i>Orthoptera</i>)	mild	no
		PA	Lehigh	Black cutworm (<i>Agrotis ipsilon</i>)	mild	no
				Grasshoppers (<i>Orthoptera</i>)	mild	no
				Japanese beetle (<i>Popillia japonica</i>)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Stink bug (<i>Pentatomidae</i>)	mild	no
				Armyworms (<i>Spodoptera</i> spp.)	mild	no
		TX	Armstrong	Aphids (<i>Aphididae</i>)	moderate	no
Armyworms (<i>Spodoptera</i> spp.)	mild			no		
Corn earworm (<i>Helicoverpa zea</i>)	mild			no		

Table A13-13. Observations of Insects Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-285-103n	HI	Kauai	Chinese rose beetle (<i>Adoretus sinicus</i>)	mild	no
	17-311-102n	PR	Salinas	Armyworms (<i>Spodoptera</i> spp.)	moderate	no
				Leafhopper (<i>Cicadellidae</i>)	mild to moderate	no
				Grasshoppers (<i>Orthoptera</i>)	moderate	no
				Aphids (<i>Aphididae</i>)	mild	no
				Corn sap beetle (<i>Carpophilus</i> spp.)	mild	no
				Corn earworm (<i>Helicoverpa zea</i>)	mild	no
				Two-spotted spider mite (<i>Tetranychus urticae</i>)	mild	no
				Corn silk fly (<i>Euxesta stigmatias</i>)	mild	no

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2009	09-033-112n	HI	Kauai	Common rust (<i>Puccinia sorghi</i>)	mild	no
2010	09-264-101n	HI	Kauai	Maize chlorotic mottle machlomovirus	mild	no
				Maize mosaic rhabdovirus	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
	10-052-101n	HI	Kauai	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild to moderate	no
		IA	Polk	Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild to moderate	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
10-284-101n	HI	Kauai	Common smut (<i>Ustilago maydis</i>)	mild to moderate	no	
2011	11-040-122n	HI	Kauai	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
	11-288-101n	PR	Ponce	Corn eyespot (<i>Aureobasidium zeae</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
2012	12-202-101n	PR	Salinas	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no
2013	12-202-101n	HI	Kauai	Common rust (<i>Puccinia sorghi</i>)	mild	no
	13-009-102n	HI	Kauai	Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
	13-294-102n	HI	Kauai	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no
		PR	Salinas	Stewart's wilt (<i>Pantoea stewartii</i>)	mild	no
				Gibberella ear (<i>Gibberella zeae</i>)	mild	no
Common rust (<i>Puccinia sorghi</i>)	mild	no				

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2014	14-002-104n	AR	Clay	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
		CA	Yolo	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
		IL	Fulton	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
			Mason	Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild to moderate	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
			McDonough	Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
		IN	Tipton	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
				Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild to moderate	no
		IA	Jasper	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
				Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	moderate	no
				Common rust (<i>Puccinia sorghi</i>)	moderate	no
			Polk	Common rust (<i>Puccinia sorghi</i>)	mild to moderate	no
				Common smut (<i>Ustilago maydis</i>)	mild to moderate	no
				Southern rust (<i>Puccinia polysora</i>)	mild to moderate	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
			Linn	Common rust (<i>Puccinia sorghi</i>)	moderate	no
			Louisa	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	moderate	no
Common rust (<i>Puccinia sorghi</i>)	mild			no		
Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no				
NE	York	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no		
		Common rust (<i>Puccinia sorghi</i>)	mild	no		
		Common smut (<i>Ustilago maydis</i>)	mild	no		

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2014	14-002-104n	TN	Obion	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
				Stewart's wilt (<i>Pantoea stewartii</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
	14-300-106n	HI	Kauai	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
		PR	Salinas	Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild	no
				Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild	no				
2015	15-012-101n	CA	Yolo	Common smut (<i>Ustilago maydis</i>)	mild to moderate	no
				Fusarium ear rot (<i>Fusarium subglutinans</i>)	mild to moderate	no
		IL	Bureau	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to severe	no
				Common rust (<i>Puccinia sorghi</i>)	mild to severe	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild to severe	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	severe	no
				Tar spot (<i>Coniothyrium phyllachorae</i>)	severe	no
				Anthraxnose (<i>Colletotrichum graminicola</i>)	severe	no
				Eye spot of corn (<i>Aureobasidium zeae</i>)	severe	no
			Champaign	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild to moderate	no
				Common rust (<i>Puccinia sorghi</i>)	moderate	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	moderate	no
			Mason	Common rust (<i>Puccinia sorghi</i>)	moderate	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
			McDonough	Common rust (<i>Puccinia sorghi</i>)	mild to moderate	no
						Common rust (<i>Puccinia sorghi</i>)

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2015	15-012-101n	IN	Tipton	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	moderate	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	moderate	no
		IA	Dallas	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
				Common rust (<i>Puccinia sorghi</i>)	mild to moderate	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	moderate	no
				Southern rust (<i>Puccinia polysora</i>)	moderate	no
			Kossuth	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
				Common rust (<i>Puccinia sorghi</i>)	mild to moderate	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild to moderate	no
				Eye spot of corn (<i>Aureobasidium zeae</i>)	moderate	no
			Polk	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild to moderate	no
				Southern rust (<i>Puccinia polysora</i>)	mild to moderate	no
				Common rust (<i>Puccinia sorghi</i>)	mild to moderate	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	moderate	no
		KS	Finney	Common smut (<i>Ustilago maydis</i>)	mild to moderate	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
		NE	York	Common smut (<i>Ustilago maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild	no
			Lancaster	Southern rust (<i>Puccinia polysora</i>)	mild	no
Common rust (<i>Puccinia sorghi</i>)	mild			no		
Common smut (<i>Ustilago maydis</i>)	mild			no		
Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild			no		
Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild			no		
Common smut (<i>Ustilago maydis</i>)	mild			no		

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?	
2015	15-012-101n	PR	Ponce	Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild to moderate	no	
				Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild to moderate	no	
				Common smut (<i>Ustilago maydis</i>)	moderate	no	
		Salinas	Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild	no		
			Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild to severe	no		
			Hale	Common rust (<i>Puccinia sorghi</i>)	mild	no	
	WI	Rock	Common rust (<i>Puccinia sorghi</i>)	mild	no		
	15-300-101n	PR	Salinas	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no	
				Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild	no	
				Bacterial stalk rot (<i>Erwinia chrysanthemi</i> pv. <i>Zeae</i>)	mild	no	
2016	16-015-103n	CA	Yolo	Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no	
		HI	Kauai	Common smut (<i>Ustilago maydis</i>)	mild to moderate	no	
		IL	Bureau	Common rust (<i>Puccinia sorghi</i>)	mild	no	
				Common smut (<i>Ustilago maydis</i>)	mild	no	
				Common rust (<i>Puccinia sorghi</i>)	mild	no	
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no	
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild to moderate	no	
				Southern rust (<i>Puccinia polysora</i>)	mild	no	
			Champaign	Common rust (<i>Puccinia sorghi</i>)	mild	no	
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild to moderate	no	
				Mason	Common rust (<i>Puccinia sorghi</i>)	mild	no
					Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild	no
		Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild		no		
		McDonough	Common rust (<i>Puccinia sorghi</i>)	mild	no		
			Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild	no		
			Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no		
			IN	Tipton	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no					
Northern corn leaf spot (<i>Bipolaris zeicola</i>)	mild	no					

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2016	16-015-103n	IA	Polk	Common smut (<i>Ustilago maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
		KS	Finney	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
				<i>Aspergillus</i> ear rot (<i>Aspergillus</i> spp.)	mild	no
			Hodgeman	Common smut (<i>Ustilago maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
				<i>Aspergillus</i> ear rot (<i>Aspergillus</i> spp.)	mild	no
		NE	York	Common smut (<i>Ustilago maydis</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
		SD	Brookings	Northern corn leaf spot (<i>Bipolaris zeicola</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
		TX	Hale	Common rust (<i>Puccinia sorghi</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
	<i>Fusarium</i> ear rot (<i>Fusarium subglutinans</i>)			mild	no	
	Common rust (<i>Puccinia sorghi</i>)			mild	no	
	WI	Rock	Eye spot of corn (<i>Aureobasidium zeae</i>)	mild	no	
Northern corn leaf blight (<i>Exserohilum turcicum</i>)			mild	no		
Gray leaf spot (<i>Cercospora zeaе-maydis</i>)			mild	no		
Common rust (<i>Puccinia sorghi</i>)			mild	no		
16-291-102n	HI	Kauai	Common rust (<i>Puccinia sorghi</i>)	mild	no	
	PR	Salinas	Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no	
			Common rust (<i>Puccinia sorghi</i>)	mild	no	

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?	
2017	16-291-102n	PR	Guayama	Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no	
				Common rust (<i>Puccinia sorghi</i>)	mild	no	
			Salinas	Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild	no	
	17-017-102n	17-017-102n	HI	Kauai	Maize stripe virus (<i>Tenuivirus</i>)	mild	no
			IL	McDonough	Common rust (<i>Puccinia sorghi</i>)	mild	no
					Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
					Northern corn leaf spot (<i>Bipolaris zeicola</i>)	mild	no
				Champaign	Common rust (<i>Puccinia sorghi</i>)	mild	no
					Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
					Bureau	Common smut (<i>Ustilago maydis</i>)	mild
			Common rust (<i>Puccinia sorghi</i>)	mild		no	
			Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild		no	
			Northern corn leaf spot (<i>Bipolaris zeicola</i>)	mild		no	
			IA	Dallas	Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
					Common rust (<i>Puccinia sorghi</i>)	mild	no
				Linn	Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
					Common rust (<i>Puccinia sorghi</i>)	mild	no
					Brown spot (<i>Physoderma maydis</i>)	moderate	no
					Northern corn leaf spot (<i>Bipolaris zeicola</i>)	mild	no
				Polk	Common rust (<i>Puccinia sorghi</i>)	mild	no
					Common smut (<i>Ustilago maydis</i>)	mild	no
			Gray leaf spot (<i>Cercospora zeaе-maydis</i>)		mild	no	
			KS	Finney	Aspergillus ear rot (<i>Aspergillus</i> spp.)	mild	no
					Common rust (<i>Puccinia sorghi</i>)	mild	no
					Common smut (<i>Ustilago maydis</i>)	mild	no
				Hodgeman	Gibberella ear (<i>Gibberella zeaе</i>)	mild	no
					Aspergillus ear rot (<i>Aspergillus</i> spp.)	mild	no
Common rust (<i>Puccinia sorghi</i>)					mild	no	
NE			York	Common smut (<i>Ustilago maydis</i>)	mild	no	
				Gibberella ear (<i>Gibberella zeaе</i>)	mild	no	
	Common smut (<i>Ustilago maydis</i>)	mild		no			
	Common rust (<i>Puccinia sorghi</i>)	mild		no			
	Southern rust (<i>Puccinia polysora</i>)	mild		no			

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-017-102n	PR	Salinas	Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild to moderate	no
				Northern corn leaf spot (<i>Bipolaris zeicola</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Southern corn leaf blight (<i>Bipolaris maydis</i>)	mild	no
			Aibonito	Bacterial leaf blight and stalk rot (<i>Pseudomonas avenae</i> subsp. <i>avenae</i>)	mild	no
				Maize stripe virus (<i>Tenuivirus</i>)	mild	no
				Northern corn leaf spot (<i>Bipolaris zeicola</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
		TN	Obion	Brown spot (<i>Physoderma maydis</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild to moderate	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
				Aspergillus ear rot (<i>Aspergillus</i> spp.)	mild	no
		TX	Hale	Fusarium ear rot (<i>Fusarium subglutinans</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
	WI	Rock	Southern rust (<i>Puccinia polysora</i>)	severe	no	
			Common rust (<i>Puccinia sorghi</i>)	severe	no	
			Common rust (<i>Puccinia sorghi</i>)	mild	no	
	17-019-101n	IL	Shelby	Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no
				Southern rust (<i>Puccinia polysora</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
IN		Boone	Common rust (<i>Puccinia sorghi</i>)	mild	no	
			Gray leaf spot (<i>Cercospora zeaе-maydis</i>)	mild	no	
IA		Audubon	Common smut (<i>Ustilago maydis</i>)	mild	no	
	Common rust (<i>Puccinia sorghi</i>)		mild to moderate	no		

Table A13-14. Observations of Diseases Present and Comparison Between DP202216 Maize and Control (continued)

Year of Planting	Permit Name	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2017	17-019-101n	KS	Pawnee	Gibberella ear (<i>Gibberella zeae</i>)	mild	no
				Aspergillus ear rot (<i>Aspergillus</i> spp.)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
		MO	Butler	Southern rust (<i>Puccinia polysora</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
		NE	York	Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild	no
				Common smut (<i>Ustilago maydis</i>)	mild	no
				Gray leaf spot (<i>Cercospora zeae-maydis</i>)	mild to moderate	no
		PA	Lehigh	Northern corn leaf blight (<i>Exserohilum turcicum</i>)	mild	no
				Common rust (<i>Puccinia sorghi</i>)	mild	no
				Diplodia ear rot (<i>Diplodia maydis</i> or <i>Diplodia macrospora</i>)	mild	no
				Fusarium ear rot (<i>Fusarium subglutinans</i>)	mild	no
	17-311-102n	PR	Salinas	Gibberella ear (<i>Gibberella zeae</i>)	mild	no
Southern corn leaf blight (<i>Bipolaris maydis</i>)				mild	no	

Table A13-15. Observations of Abiotic Stressors and Comparison Between DP202216 Maize and Control

Year of Planting	Permit Name	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	IA	Polk	Above average rainfall, hail, and wind	mild	no
2011	11-040-122n	IA	Polk	Early season above average rains and high winds	mild	no
2014	14-002-104n	IL	Mason	Wind event (green snap, root lodging)	mild	no
			McDonough	Wind and hail damage	moderate	no
		IA	Polk	Root lodging and brittle snap due to early season above average rains and high winds	mild	no
		NE	York	Above average rainfall, hail, and wind	mild to severe	no
2015	15-012-101n	IL	Bureau	High rainfall amounts has caused saturated soil conditions	mild	no
			Champaign	Wet soil conditions most of June	moderate	no
			McDonough	High winds	mild	no
		IN	Tipton	High rainfall amounts has caused saturated soil conditions	mild	no
		IA	Dallas	High rainfall amounts has caused saturated soil conditions	moderate	no
			Polk	Root lodging due to high wind and rain events	mild	no
		NE	York	High rainfall amounts has caused saturated soil conditions	mild	no
		TX	Hale	Drought stress	severe	no
WI	Rock	Above average rainfall, hail, and wind	mild	no		
2016	16-015-103n	IL	Bureau	Greensnap, root lodging, and stalk lodging present due to multiple wind events.	moderate	no
			Champaign	High winds brittle and root lodging	severe	no
		IA	Polk	Root lodging due to high winds and heavy rain	moderate	no
		KS	Hodgeman	heavy rains and high winds that have caused some plants to root lodge and brittle snap	mild	no
		TX	Hale	High Wind and Hail	moderate	no
2017	17-017-102n	IN	Tipton	Heavy rain and wind	moderate	no
		IA	Polk	Hail and wind damage	mild	no
		KS	Hodgeman	Drought stress	mild to moderate	no
		TX	Hale	Drought stress	moderate	no
	17-019-101n	IN	Boone	Drought stress	mild	no
		IA	Audubon	High temperatures and wind	mild	no
		NE	York	Hail and wind damage	mild	no
		PA	Lehigh	Wind damage	mild	no
TX	Armstrong	Wind damage	mild	no		

Appendix 14. Mode of Action Summary – DP202216 Maize

Introduction

DP-202216-6 (hereafter DP202216) maize is genetically engineered to increase and extend the expression of a native maize transcription factor (ZMM28). The expression of ZMM28 protein in DP202216 maize plants leads to increased grain yield potential through [CBI DELETED]. DP202216 maize also contains the *mo-pat* gene that encodes the PAT protein, which confers resistance to glufosinate-ammonium herbicides. CBI DELETED

This background document characterizes the increased grain yield potential of DP202216 plants via measurement of relevant plant phenotypes. None of the information or data presented is related to the safety or nutritional wholesomeness of DP202216 maize. DP202216 maize has been previously analyzed for safety by comparing the compositional characteristics of the edible portions of DP202216 maize plants to those of near-isoline controls and conventional maize lines, performing in-depth molecular characterization, and analyzing introduced (ZMM28 and PAT) protein safety. All safety studies conclude that DP202216 maize is as safe as conventional maize.

The *zmm28* Gene in DP202216

The *zmm28* gene encodes a [CBI DELETED] transcriptional factor. The maize native *zmm28* gene begins expression at the V6 growth stage and peaks at the V11 stage, and continues through grain fill stages. In contrast to the *zmm28* gene expression in conventional maize, the *zmm28* gene in DP202216 maize begins expression in early plant growth stages (V2-V5).

ZMM28 Protein Background Information

Plant [CBI DELETED] proteins, such as ZMM28, are a family of transcription factors which bind to specific DNA sequences and regulate gene expression. This family of proteins is found across plant species and is thought to play an important role in developmental control and signal transduction in plants [CBI DELETED].

[CBI DELETED] genes encode homeotic transcription factors which are characterized by having a highly-conserved DNA-binding domain, called the [CBI DELETED] at the N-terminus. They comprise a multigene family and are highly conserved among fungi, animals and plants. The [CBI DELETED] genes have been classified into two groups: type I and type II [CBI DELETED]. The type I [CBI DELETED] proteins in plants contain a [CBI DELETED]. Plant-based Type II [CBI DELETED] genes are considered [CBI DELETED] genes of plants. This refers to their conserved domain structure, where the [CBI DELETED]

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[] proteins cause a conformational change of the DNA (bending) upon binding, via heterodimerization, which once complete with transcriptional co-factors, initiates target gene transcription [].

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The *zmm28* gene encodes the ZMM28 protein consisting of 251 amino acids. Sequence analysis reveals that it is a [] transcriptional factor which contains [

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DP202216 Maize ZMM28 Protein

Subcellular localization experiments using fluorescently tagged ZMM28 protein have been conducted in control maize and DP202216 maize plants. Results show localization to the nucleus in both control and DP202216 plants, indicating proximity to chromosomes in order to initiate transcription.

[] transcription factors form homo- or hetero-dimers that bind [] which modulate target gene expression. Yeast two hybrid (Y2H) screening and subsequent biomolecular fluorescence complementation (BiFC) experiments identified potential [] protein interaction partners. The protein partners of ZMM28 are members of the []. As the proteins interact, this promotes DNA bending and subsequent transcription of target genes.

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Identification of direct target genes in DP202216 plants occurred via transcript analysis and identification of [] binding motifs. Gene ontology enrichment further enriched the dataset and identified genes involved in [

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] as targets of the

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ZMM28 transcription factor. These results are consistent with measured phenotypes of DP202216 plants and suggest that [

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Yield Improvement

Maize yield has consistently improved since the evolution of the hybrid maize era (from approximately 1939 to present day) and is likely due to selection of physiological factors attributed to grain dry matter partitioning (Lee and Tollenaar, 2007). Much of this improvement is likely due to changes in processes influencing dry matter production, or “source” capacity, and the efficient inclusion of source products to the developing grain, or “sink” components. Positive changes to leaf canopy architecture (increased leaf area, leaf angles), delayed senescence (or “stay green”) have been noted to increase source potential, while lengthened grain fill and improved embryo health have positively affected sink capacity.

Until now, these improvements have been made via selection of desirable traits, namely yield increase, through conventional breeding. Breeding populations have utilized divergent gene pools, recurrent selection of desired phenotypes, and a gradual increase of maize planting densities to identify maize lines optimized for increased grain yield. Recent advances in biotechnology allow for further increase in grain yield by modifying genes that contribute to the source capacity of maize plants, while keeping sink capacity in balance.

Source and Sink

The processes which produce grain assimilates are known as “source” components, and the tissues which accept these products are “sink” components. In maize, source tissues are green tissues that actively photosynthesize to produce dry matter for sink tissues to accumulate. It is beneficial to keep source and sink capacities in balance, as an imbalance causes undesirable phenotypes (purpling of stems and leaves in source overproduction, and premature senescence of leaves and stalks in overwhelmed sink tissues).

DP202216 maize was produced by modern biotechnology methods, using *Agrobacterium* transformation of T-DNA fragment containing the *zmm28* and *pat* genes. Plants containing the DP202216 insert were selected based on the presence of the genes of interest, but were also evaluated for desired phenotypes. Under the conditions measured, DP202216 plants do not show signs of source and sink imbalance. Stems and leaf tissue do not show purple coloration nor early senescence of leaves and stalk. The lack of these undesirable phenotypes indicates the likelihood that source and sink components are in balance.

DP202216 Maize Plant Physical Characterization

A series of physical measurements have been collected across multi-year, broad acreage field trials to identify potential growth parameters in DP202216 plants that could be responsible for an increase in grain yield potential. The extended and increased *zmm28* gene expression enhanced early seedling growth, leaf biomass and total leaf area. Plant height of DP202216 was significantly greater than that of controls from V2 to V7, averaged across all tested hybrids. [

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DP202216 Maize Photosynthesis

Photosynthesis is a critical factor in determining crop yield and thus, measurements were collected to determine if photosynthesis was altered in DP202216 maize. Photosynthesis, expressed as CO₂ exchange rate (CER) and photosynthetic electron transport rate (ETR), was measured from field-pot grown DP202216 plants in two hybrid backgrounds together with their controls at the V11 growth stage. [

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Overall Assessment of DP202216 Enhanced Grain Yield Potential

Enhanced grain yield potential has been achieved in DP202216 maize plants (relative to control plants) using a genetic engineering approach. Based on physiological, biochemical and molecular characterization, it is likely that the molecular action of the *zmm28* gene in yield enhancement is

DuPont Pioneer
DP202216 Maize

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leading to enhanced grain yield potential.

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