Application to Amend the Food Standards Code – Food Produced Using Gene Technology

OECD Unique Identifier - DP-2Ø2216-6

DP202216 Maize

Submitting company:

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Submitted by:

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Summary

Corteva Agriscience is a publicly traded, global pure-play agriculture company that provides farmers around the world with the most complete portfolio in the industry - including a balanced and diverse mix of seed, crop protection and digital solutions focused on maximizing productivity to enhance yield and profitability. With some of the most recognized brands in agriculture and an industry-leading product and technology pipeline well positioned to drive growth, the company is committed to working with stakeholders throughout the food system as it fulfils its promise to enrich the lives of those who produce and those who consume, ensuring progress for generations to come. Corteva Agriscience became an independent public company on June 1, 2019 and was previously the Agriculture Division of DowDuPont. More information can be found at www.corteva.com.

Dow AgroSciences Australia, member of Corteva Agriscience group of companies, is submitting this application to FSANZ to vary the Code to approve uses of maize (*Zea mays L.*) event DP-2Ø2216-6 (referred to as DP202216 maize), a new food produced using gene technology.

DP202216 maize was genetically modified to increase and extend expression of the *zmm28* gene relative to the native *zmm28* gene expression. Both the introduced and native *zmm28* genes encode the *ZMM28* protein, a MADS-box transcription factor. The increased and extended expression of the *ZMM28* protein results in plants with an enhanced grain yield potential. DP202216 maize also contains the phosphinothricin acetyltransferase (PAT) protein, which confers tolerance 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 several approved events across several different crops that are currently in commercial use.

This application presents information supporting the safety and nutrition of DP202216 maize. The molecular characterization analyses conducted on DP202216 maize demonstrated that the introduced genes are integrated at a single locus, stably inherited across multiple generations, and segregate according to Mendel's law of genetics. The *ZMM28* protein is endogenous to maize, including sweet corn, and is present in food. The introduced *ZMM28* protein in DP202216 maize is identical to the native *ZMM28* protein in DP202216 maize and to the *ZMM28* protein in non-genetically modified (non-GM) maize. A compositional equivalence assessment demonstrated that the nutrient composition of DP202216 maize grain is comparable to that of non-GM maize.

Overall, DP202216 maize, containing the *ZMM28* and PAT proteins is as safe and nutritious as non-GM maize varieties for food and feed uses.

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Checklist for General requirements

Check	Mandatory requirements
	A Form of application
✓	*☐ Application in English* *☐ Executive Summary (separated from main application electronically)* *☐ Relevant sections of Part 3 clearly identified* *☐ Pages sequentially numbered* *☐ Electronic copy (searchable)* *☐ All references provided*
✓	B Applicant details
✓	C Purpose of the application
	D Justification for the application
✓	*☐ Regulatory impact information* *☐ Impact on international trade*
	E Information to support the application
✓	*☐ Data requirements*
	F Assessment procedure
✓	*☐ General* *☐ Major* *☐ Minor* *☐ High level health claim variation*
	G Confidential commercial information
✓	*☐ CCI material separated from other application material* *☐ Formal request including reasons* *☐ Non-confidential summary provided*
	H Other confidential information
✓	*☐ Confidential material separated from other application material* *☐ Formal request including reasons*
	I Exclusive Capturable Commercial Benefit
✓	*☐ Justification provided*
	J International and other national standards
✓	*☐ International standards* *☐ Other national standards*
✓	K Statutory Declaration
	L Checklist/s provided with application
✓	*☐ 3.1.1 Checklist* *☐ All page number references from application included* *☐ Any other relevant checklists for Chapters 3.2–3.7*

Checklist for foods produced using gene technology (3.5.1)

Check	Mandatory requirements
✓	A.1 Nature and identity
✓	A.2 History of use of host and donor organisms
✓	A.3 Nature of genetic modification
✓	B.1 Characterisation and safety assessment
✓	B.2 New proteins
X	B.3 Other (non-protein) new substances
X	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
✓	B.5 Compositional analyses
✓	C Nutritional impact of GM food
✓	D Other information

Statutory Declaration

Statutory Declarations Act 1959 1

I, [redacted]; Level 9, 67 Albert Ave, Chatswood, NSW, 2067; Regulatory and Stewardship Manager;

make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required

2. the information provided in this application is true to the best of my knowledge and belief

3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

[redacted]

Declared at Chatswood on 4 of December 2019

Before me, [redacted]

[redacted]

Legal Practitioner
C/- Level 9, 67 Albert Ave
Chatswood NSW 2067

General Information on the Application

1. Purpose of the application

Dow AgroSciences Australia Pty. Ltd., member of Corteva Agriscience group of companies (herein referred to as Corteva Agriscience), has developed DP202216 maize (OECD Unique Identifier DP-2Ø2216-6), a new event that has been transformed with a single genetic construct expressing the *ZMM28* and PAT proteins.

As a result of this application, Corteva Agriscience seeks an amendment of Standard 1.5.2 by inserting: food derived from DP202216 maize line, into column 1 of the Table to clause 2, after the last entry.

2. Justification for the application

a. Need for the Proposed Change

Corteva Agriscience have developed the new maize line DP202216, which is being commercialized in accordance with the ETS Product Launch Stewardship Guidance and in compliance with Corteva polices regarding stewardship of those products. A component of this process is ensuring that the appropriate regulatory approvals have been obtained globally.

b. Advantage of the Genetically Modified Food

DP202216 maize was genetically modified 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 MADS-box transcription factor. The increased and extended expression of the *ZMM28* protein results in plants with an enhanced grain yield potential (Wu, 2019). The PAT protein confers tolerance to glufosinate-ammonium, the active ingredient in phosphinothricin herbicides.

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 gain yield complements the selection of genes by breeding.

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

c. Potential Impact on Trade

Corteva Agriscience is a member of Excellence Through Stewardship™ (ETS). Corteva products are commercialized in accordance with the ETS Product Launch Stewardship Guidance and in compliance with Corteva polices regarding stewardship of those products. Dossiers are being submitted to the regulatory authorities of trade partners for import clearance may include Canada, Japan, Korea, Taiwan, European Union, ANZ, South Africa, Brazil, Argentina, Mexico and Colombia.

d. Costs and Benefits for Industry, Consumers and Government

Corteva Agriscience acknowledges that the proposed amendment to the Standard will likely result in an exclusive capturable commercial benefit being accrued to the parent company as defined in Section 8 of the *FSANZ Act*.

Most of the sweet corn consumed in Australia is grown domestically. Domestic production of corn in Australia and New Zealand is supplemented by import of a small amount of corn-based products usually frozen or canned, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand (www.grdc.com.au). Although not requiring a FSANZ approval for livestock feed, from time to time, mainly during periods of drought where local supply of feed grain is limited, maize is imported from the United States for use as stock feed, predominantly in the pig and poultry markets. This variation to the Standard would permit the import and use of food derived from DP202216 maize.

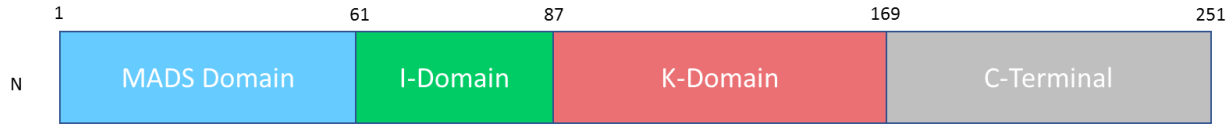
A. Technical information on the food produced using gene technology

A.1 Nature and identity of the genetical modified food

a. Description of the GM organisms, nature and purpose of the genetic modification

DP202216 maize was generated by the insertion of the *zmm28* gene which was isolated from maize and the maize-optimized phosphinothricin acetyltransferase gene (*mo-pat*) which was isolated from Streptomyces viridochromogenes. The *zmm28* gene cassette in DP202216 maize 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, a MADS-box transcription factor. The increased and extended expression of the *ZMM28* protein results in plants with an enhanced grain yield potential. The PAT protein, encoded by the *mo-pat* gene, confers tolerance to the herbicidal active ingredient glufosinate.

The *ZMM28* protein, encoded by the *zmm28* gene, is a MADS-box transcription factor (Münster et al., 2002). MADS-box transcription factors bind to specific DNA sequences termed the CArG-box as homo- or heterodimers, or even multimers to regulate gene expression (Kaufmann et al., 2005; Smaczniak et al., 2012). The *ZMM28* transcription factor is an MIKC protein which contains an N-terminal MADS domain involved in DNA-binding, followed by an Intervening (I) region and a Keratin-like (K) box which are both involved in DNA binding and protein-protein interactions, and a C-terminal domain that is integral to activity and ternary complex formation (Kaufmann et al., 2005; Theissen et al., 2000). The MIKC structure and the corresponding *ZMM28* amino acid sequence are illustrated (**Figure 1**).

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  1 MGRGPVQLRR IENKINRQVT FSKRRNGLLK KAHEISVLCD AEVALIVFST KGKLYEYSSH
 61 SSMEGILERY QRYSFEERAV LNPSIEDQAN WGDEYVRLKS KLDALQKSKR QLLGEQLSSL
121 TIKELQQLEQ QLDSSLKHIR SRKNQLMFDS ISALQKKEKA LTDQNGVLQK FMEAEKEKNK
181 ALMNAQLREQ QNGASTSSPS LSPPIVPDSM PTLNIGPCQH RGAAESESEP SPAPAQANRG
241 NLPPWMLRTV K
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Figure 1. ZMM28 Transcription Factor Domain Structure and Protein Sequence

b. GM Organism Identification

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants", this event has an OCED identifier of DP-2Ø2216-6, also referred to as DP202216.

c. Food Identity

Maize event DP202216 is at pre-commercialization stage and has not yet been assigned a commercial product name. In the event that maize event DP202216 is commercialized as a stand-alone product, Corteva Agriscience will provide Food Standards Australia New Zealand the commercial name once it is available. The introduced traits of yield and herbicide-tolerance in DP202216 maize are not intended to change any of the end-use characteristics

of maize grain and the commercial introduction of maize hybrid containing event DP202216 is not anticipated to change the usage and consumption patterns of maize grain. It is anticipated that following commercialization, any food containing maize products may contain material derived from DP202216 maize.

d. Products containing the food or food ingredients.

Refer to the OECD Consensus Document on Compositional Considerations for New Varieties of Maize (Zea mays): Key Food and Feed Nutrients, Anti-nutrients and Secondary Plant Metabolites (2002), for the following aspects of the food uses of maize:

- Production of maize for food and feed
- Processing of maize
- Wet Milling
- Dry Milling
- Masa Production
- Feed Processing

The majority of grain and forage derived from maize is used for animal feeds. Less than 10% if maize grain is processed for human food products. Maize grain is also processed into industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet-milling to produce starch and sweetener products. In addition to milling, maize germ can be processed to obtain maize oil.

Domestic production of maize in Australia (ca. 440,000t) and New Zealand is supplemented by import of a small amount of maize-based products, largely as high-fructose maize syrup, which is not currently manufactured in either Australia or New Zealand. Such products are processed into breakfast cereals, baking products, extruded confectionery and maize chips. Other maize products such as maize starch are also imported. This is used by the food industry for the manufacture of dessert mixes and canned foods (www.grdc.com.au).

A.2 History of use of the host and donor organisms

a. Donor Organism

***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; today mainly hybrids are grown (OECD, 2002). Worldwide production of maize was about 1033 million tons in 2017 (FAO, 2012; USDA-FAS, 2018).

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 and produces the tripeptide phosphinothricyl L alanyl-L-alanine, which was developed as a non-selective herbicide. The *mo-pat* gene, encoding the phosphinothricin acetyltransferase (PAT) enzyme, confers tolerance to the phosphinothricin herbicide application (OECD, 1999).

Other Donor Organisms

The other donor organism, potato (*Solanum tuberosum*), was used as a source for the regulatory sequence of the *pin*II terminator that is not expressed in the transformed plant. Since this sequence does not encode any expressed products in DP202216 maize, its donor organism is of little relevance to assessing potential toxicity or potential allergenicity. *Zea mays* is the donor of the *zm-gos2* and *ubi*ZM1 promoters and intron regulatory regions.

Please refer to Part C, section 4 and 5 of this dossier for information relating to the potential allergenicity and toxicity of the expressed protein.

b. Host Organism

Maize is extensively cultivated worldwide and has a long history of safe use. Maize grain and maize-derived products represent staple food and feed for a large portion of the global population (CFIA, 1994). No significant

toxicity or allergenicity has been ascribed to any food or feed uses of maize, and maize has been described as a food that is likely to have low allergenicity (OECD, 2002). Maize is not included in the list of food allergy indications of the US Food and Drug Administration (FDA) (US-FDA, 2006).

Taxonomy

- Family name: *Poaceae* (Gramineae)
- Genus: *Zea*
- Species: *Z. mays L.*
- Subspecies: *Zea mays* ssp*. mays L*
- Common name: Maize; corn

Maize is a diploid species with a chromosome number of 2n = 2x = 20 and is a domesticated species of the tribe *Maydeae* and the grass family, *Poaceae*. The closest relatives to the genus *Zea* are grasses in the genus *Tripsacum* (OECD, 2003). Within the genus *Zea*, there are five species, including *Zea mays. Z. mays* contains four subspecies, including *Zea mays* ssp. *mays*, which is the only domesticated taxon (maize). The other three subspecies of *Z. mays* are called teosintes (OECD, 2003) including *huehuetenangensis, mexicana,* and *parviglumis*.

Morphology

Biology documents on unmodified maize have been published by the OECD (OECD, 2003). Maize is a tall annual grass consisting of a stalk with overlapping sheaths and broad leaves growing alternately around the stalk. Plants have one or more female flowers, consisting of silk on a thickened axis (cob), located midway on the stalk. Plants also have male flowers, consisting of the tassel at the top of the plant, which release pollen. Maize plants reproduce sexually, during which an individual silk must become pollinated, and fertilization must take place to produce one maize kernel. Kernels develop in 8 to 16 rows along the cob, which is surrounded by a layer of protective leaves called a husk (OECD, 2003).

Centre of Origin

The Meso-American region (middle South Mexico and Central America) is recognized as the centre of origin for maize (OECD, 2003).

Natural Habitat and Generation Time

Maize is grown over a wide range of climatic conditions and is well-suited for warm, temperate climates. The majority of maize is produced between latitudes 30 and 55 degrees, with a relatively small amount grown at latitudes higher than 47 degrees (Shaw, 1988). The greatest maize production occurs where the warmest month isotherms range between 21 °C and 27 °C and the freeze-free season lasts 120 to 180 days (Shaw, 1988). Survival and reproduction of maize are limited by extreme environmental conditions (heat stress, frost, drought, excessive rainfall, etc.) (Shaw, 1988). Maize is typically not cultivated in areas where the mean mid-summer temperature is < 19 ºC (66 ºF) or where the average night temperature falls much below 13 ºC (55 ºF). Maize yield is also susceptible both to excess water and low moisture stress. There is no upper limit of rainfall for growing maize, although excess rainfall will decrease yields (Shaw, 1988).

The maize life cycle ranges from as short as 10 weeks to as long as 48 weeks covering the period of seedling emergence to maturity (OECD, 2003; Shaw, 1988). The duration of the maize life cycle depends on the maize variety and environmental conditions in which it is grown (OECD, 2003).

Mode of Reproduction and Dispersal

Maize plants reproduce sexually with both male (tassels) and female (silk) reproductive organs present on each plant (monoecious species). Pollen, produced by the tassel, can pollinate silks from the same plant (self-fertilization) or can pollinate silks from neighbouring plants (cross fertilization). Both self-fertilization and cross-pollination are influenced by plant proximity, pollen dispersal, and pollen viability. Normally, approximately 95% of ovules are cross-pollinated from plants in the immediate vicinity, and 5% are self-pollinated (Sleper and Poehlman, 2006).

Wind-dispersal is the primary method by which pollen is carried to fertilize other maize plants (Galinat, 1988; Raynor et al., 1972; Russell and Hallauer, 1980); however, viable maize pollen generally does not travel long distances (95-99% of maize pollen will be deposited within 30 meters of the source (Devos et al., 2005; OECD, 2003). Insects such as bees, beetles, flies, and leafhoppers do enable pollen dispersal; however, the magnitude of that dispersal pathway is limited (Andersson and de Vicente, 2010).

Maize is highly domesticated, and the structure of the ear (cob and seeds enclosed in husk) limits seed dispersal from occurring naturally in the environment (Andersson and de Vicente, 2010; CFIA, 1994; OECD, 2003; Raybould et al., 2012). Without human or animal aid, seed dispersal is limited to within a meter or so of the plant. Neither animal dispersal nor unintentional human dispersal distributes enough seed to be considered significant (Andersson and de Vicente, 2010).

Outcrossing Rate (Intra-Specific and Inter-Specific Crosses)/Gene Flow

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

The risk of gene flow and introgression of transgenes from DP202216 maize into other varieties of cultivated maize is unlikely. This application seeks authorization of DP202216 maize for import for food and feed uses, and commercialization by Corteva Agriscience for cultivation of DP202216 in Australia is not currently planned.

Survival, Dormancy, and Weediness/Invasiveness

Maize is grown over a wide range of climatic conditions and is well-suited for warm, temperate climates (OECD, 2003). Survival and reproduction of maize are limited by extreme environmental conditions (heat stress, frost, drought, excessive rainfall, etc.) (Shaw, 1988). Populations of maize are unlikely to survive outside managed agricultural environments. Although plants may occasionally grow in uncultivated fields or occur as volunteers, maize generally does not sustain reproduction outside of cultivation (CFIA, 1994). Maize seeds are the only survival structures, and natural regeneration of maize from vegetative tissue is not known to occur.

Maize seeds show poor dormancy (CFIA, 1994) and generally only survive under favourable climatic conditions. Maize is an annual plant that lacks seed dormancy which limits survival from one growing season to the next (Andersson and de Vicente, 2010; CFIA, 1994).

Conventional maize is well established as having low weediness and invasiveness potential, is highly domesticated and unlikely to establish itself in self-sustaining populations outside of cultivation and is a poor competitor with native vegetation and lack of seed dormancy adds to its inability to establish sustainable feral populations (Raybould et al., 2012). Additionally, maize has no history of weediness or invasiveness in either natural or managed agricultural systems (Raybould and Wilkinson, 2005).

Maize is a commonly cultivated crop around the world, and its biology and history of safe use demonstrate that the unmodified organism is safe for human and animal consumption.

A.3 The nature of the genetic modification

a. Transformation Method

DP202216 maize was created by Agrobacterium-mediated transformation with plasmid PHP40099 (**Figure 2**; **Table 1**.). The transfer DNA (T DNA) region from plasmid PHP40099 (**Figure 2**; **Table 2**) contains two gene cassettes.

The first gene cassette (*zmm28* gene cassette) contains the *zmm28* gene from Zea mays encoding the *ZMM28* protein, a MADS-box transcription factor (Münster et al., 2002; Pařenicová et al., 2003). The increased and extended expression of the *ZMM28* protein results in plants with an 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 (U.S. Patent 9115203) 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).

The second gene cassette (*mo-pat* gene cassette) contains a maize-optimized version of the phosphinothricin acetyltransferase gene (*mo-pat*) from Streptomyces viridochromogenes (Wohlleben et al., 1988). The *mo-pat* gene expresses the phosphinothricin acetyltransferase (PAT) enzyme that confers tolerance to phosphinothricin. The PAT protein is 183 amino acids in length and has a molecular weight of approximately 21 kDa. Expression of the *mo-pat* gene is controlled by the promoter 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).

Proprietary inbred line PH17AW was transformed with plasmid PHP40099 to produce DP202216 maize. Immature maize embryos were harvested from a surface-sterilized ear of PH17AW maize approximately 8-11 days after pollination and inoculated with *Agrobacterium tumefaciens* strain LBA4404 (Zhao et al., 2001) containing plasmid PHP40099. *Agrobacterium tumefaciens* strain LBA4404 is a disarmed strain that does not contain tumour-inducing factors; 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 tissue. After 3-6 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 a 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.

b. Construct and Transformation Vectors

Table 1. Description of Genetic Elements in Plasmid PHP40099 [redacted] 2018)

Region	Location on Plasmid (bp to bp)	Genetic Element	Size (bp)	Description
T-DNA	1 – 7,470		7,470	See Table 2 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)	*spc*	789	Spectinomycin resistance gene from bacteria (Fling *et al.*, 1985)
	10,557 – 10,926 (complementary)	colE1 *ori*	370	Origin of replication region from Escherichia coli (Tomizawa *et al.*, 1977)
	12,022 – 12,035	*cos*	14	Cohesive ends from lambda bacteriophage DNA (Komari *et al.*, 1996)
	13,740 – 14,390 (complementary)	*tetR*	651	Tetracycline resistance regulation gene from bacteria (Komari *et al.*, 1996)
	14,496 – 15,695	*tetA*	1,200	Tetracycline resistance gene from bacteria (Komari *et al.*, 1996)
	16,968 – 18,116 (complementary)	*trfA*	1,149	Trans-acting replication gene from bacteria (Komari *et al.*, 1996)
	21,930 – 22,041	*ori*T	112	Origin of transfer region from bacteria (Komari *et al.*, 1996)
	23,881 – 30,151 (complementary)	*ctl*	6,271	Central control operon region from bacteria (Komari *et al.*, 1996)
	31,159 – 31,869 (complementary)	*ori*V	711	Origin of replication region from bacteria (Komari *et al.*, 1996)
Ti Plasmid Backbone	**32,357 – 47,173**	**Includes Elements Below**	**14,817**	**Virulence (*vir*) gene and intergenic regions from the *Agrobacterium tumefaciens* Ti plasmid** (Komari *et al.*, 1996)
	32,670 – 33,113 (complementary)	*vir*D1	444	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	33,382 – 34,076	*vir*C1	695	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	34,079 – 34,687	*vir*C2	609	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	34,798 – 35,601 (complementary)	*vir*G	804	Virulence gene from *Agrobacterium tumefaciens* important for T-DNA insertion into genome
	35,733 – 45,168 (complementary)	*vir*B	9,436	Virulence operon region from *Agrobacterium tumefaciens* 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)	colE1 *ori*	370	Origin of replication region from *Escherichia coli* (Tomizawa *et al.*, 1977)
	48,931 – 48,944	*cos*	14	Cohesive ends from lambda bacteriophage DNA (Komari *et al.*, 1996)

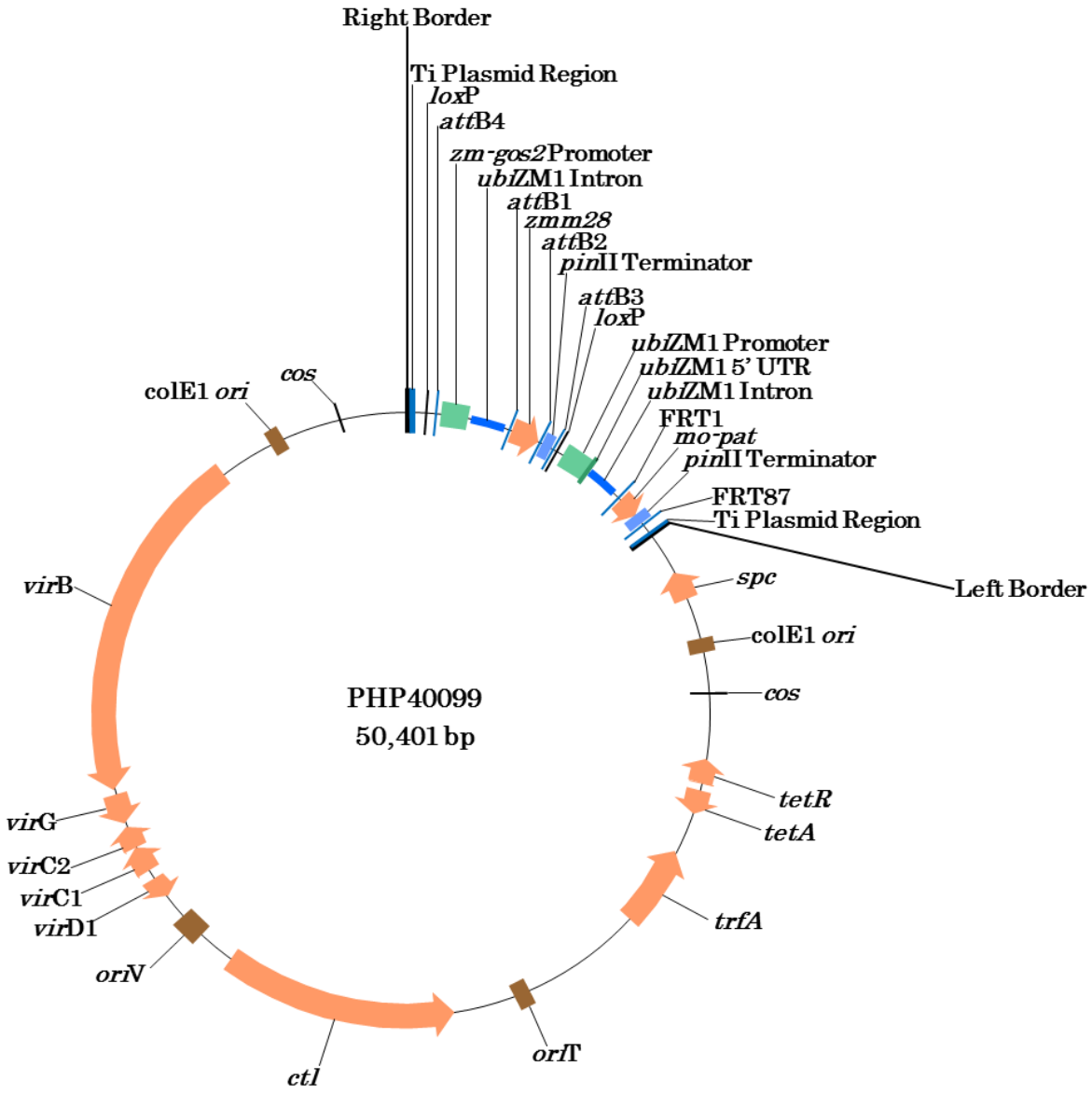


Figure 2. Map of Plasmid PHP40099

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 1**.

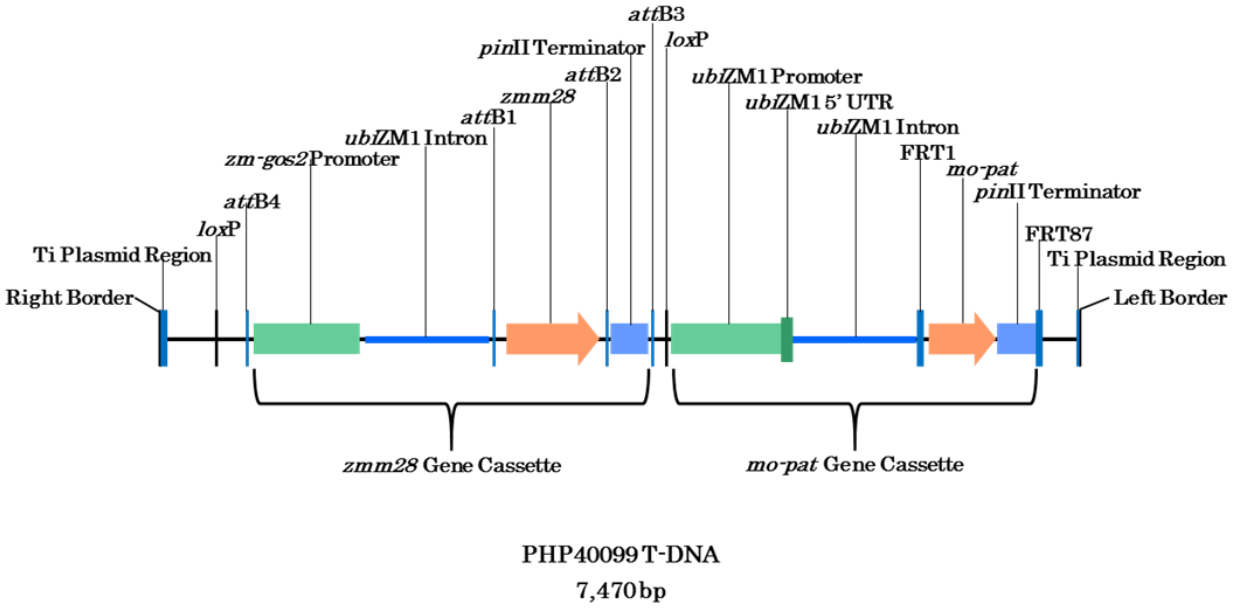


Figure 3. Map of the T-DNA Region from Plasmid PHP40099

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 2**.

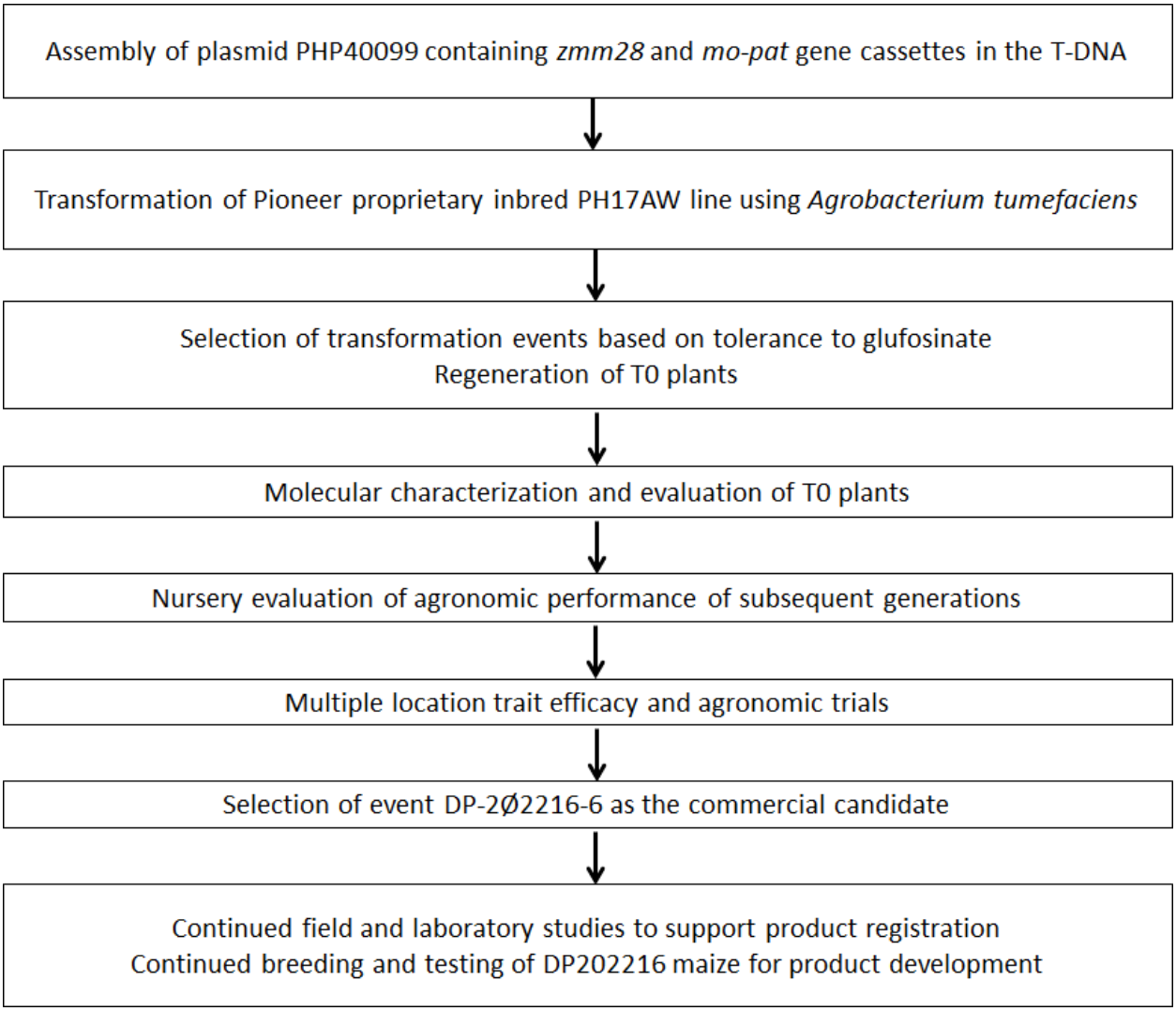
Table 2. Description of Genetic Elements in T-DNA Region of Plasmid PHP40099

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
***zmm28* Gene Cassette**	1 – 25	Right Border (RB)	25	T-DNA Right Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari *et al.*, 1996)
	26 – 177	Ti Plasmid Region	152	Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari *et al.*, 1996)
	178 – 435	Intervening Sequence	258	DNA sequence used for cloning
	436 – 469	*lox*P	34	Bacteriophage P1 recombination site recognized by Cre recombinase (Dale and Ow, 1990)
	470 – 698	Intervening Sequence	229	DNA sequence used for cloning
	699 – 719	*att*B4	21	Bacteriophage lambda integrase recombination site (Cheo *et al.*, 2004)
	720 – 753	Intervening Sequence	34	DNA sequence used for cloning
	754 – 1,613	*zm-gos2* Promoter	860	Promoter region from the *Zea mays* translation initiation factor *gos2* gene (U.S. Patent 9115203)
	1,614 – 1,654	Intervening Sequence	41	DNA sequence used for cloning
	1,655 – 2,667	*ubi*ZM1 Intron	1,013	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	2,668 – 2,707	Intervening Sequence	40	DNA sequence used for cloning
	2,708 – 2,731	*att*B1	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	*zmm28*	857	MADS-domain transcription factor gene region from *Zea mays* including 5' and 3' untranslated regions (UTR) (Münster *et al.*, 2002; Pařenicová *et al.*, 2003) 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	*att*B2	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

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
***zmm28* Gene Cassette**	3,660 – 3,967	*pin*II Terminator[1]	308	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An *et al.*, 1989; Keil *et al.*, 1986)
	3,968 – 3,997	Intervening Sequence	30	DNA sequence used for cloning
	3,998 – 4,018	*att*B3	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	*lox*P	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
***mo-pat* Gene Cassette**	4,145 – 5,044	*ubi*ZM1 Promoter	900	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	5,045 – 5,127	*ubi*ZM1 5' UTR	83	5’ untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	5,128 – 6,140	*ubi*ZM1 Intron	1,013	Intron region from the *Zea mays* 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 *Saccharomyces cerevisiae* (Proteau *et al.*, 1986)
	6,217 – 6,242	Intervening Sequence	26	DNA sequence used for cloning
	6,243 – 6,794	*mo-pat*	552	Maize-optimized phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988)
	6,795 – 6,801	Intervening Sequence	7	DNA sequence used for cloning
	6,802 – 7,112	*pin*II Terminator	311	Terminator region from the *Solanum tuberosum* (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 *Saccharomyces cerevisiae* (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 *Agrobacterium tumefaciens* Ti plasmid (Komari *et al.*, 1996)
	7,446 – 7,470	Left Border (LB)	25	T-DNA Left Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari *et al.*, 1996)

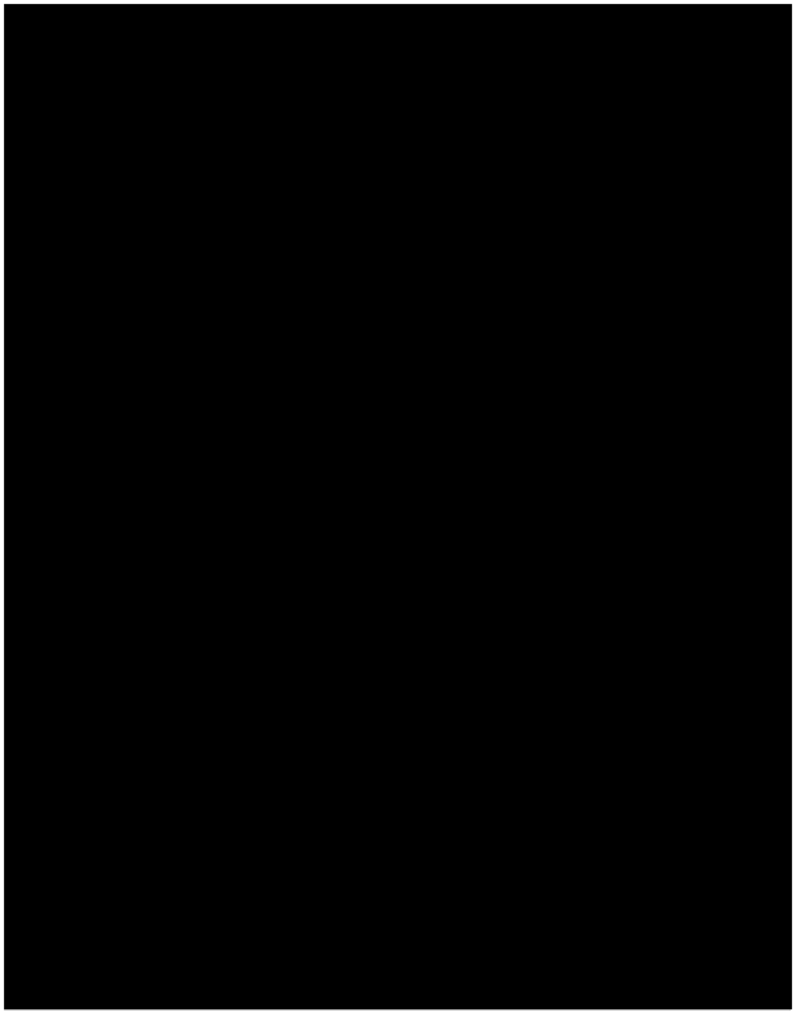
[1] This copy of the pinII terminator is 3 bp shorter at the 5’ end than the other pinII terminator in this vector.

Refer to **Figure 4** for a schematic overview of the transformation and event development process for DP202216 maize. The subsequent breeding of DP202216 maize proceeded as indicated in **Figure 5** to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial lines.



Analysis	DP202216 Maize Generations Used	Control
Southern-by-Sequencing	T1	PH17AW
Detection Method	T1	
Stability in Five Generations Using Southern Blot Analysis	T1, T2, BC1F1, BC3F3, BC3F6	PH17AW and PHR1J
Mendelian Inheritance	T2, F1(PH17AW/PHR1J), BC1F1, BC3F3, BC3F6)	Not Applicable
Sequence Characterization of Insert and Flanking Genomic Region	F1(PHR1J/PHW2Z)	Not Applicable
Protein Expression	F1(PHR1J/PHW2Z)	Not Applicable
Composition	F1(PHR1J/PHW2Z)	PHR1J/PHW2Z

Figure 4. Schematic Diagram of the Development of DP202216 Maize



Analysis	DP202216 Maize Generations Used
Southern-by-Sequencing	T1
Detection Method	F1(PHR1J/PHW2Z)
Stability in Five Generations Using Southern Blot Analysis	T1, T2, BC1F1, BC3F3, BC3F6
Mendelian Inheritance	T2, F1(PH17AW/PHR1J), BC1F1, BC3F3, BC3F6)
Sequence Characterization of Insert and Flanking Genomic Region	F1(PHR1J/PHW2Z)
Protein Expression	F1(PHR1J/PHW2Z)
Composition	F1(PHR1J/PHW2Z)

Figure 5. Breeding Diagram for DP202216 Maize and Generations Used for Analyses

Bacteria used for manipulation

A standard lab strain of Agrobacterium tumefaciens (strain LBA4404) was used for all vector manipulations and for amplification of the plasmid DNA (PHP40099) that was used for the transformation.

Gene Construct and Vectors

DP202216 maize was generated by the insertion of the *zmm28* gene which was isolated from maize and the maize-optimized phosphinothricin acetyltransferase gene (*mo-pat*) which was isolated from *Streptomyces viridochromogenes*. Please refer to **Table 1** for a summary of the genetic elements; **Figure 2** for a map of Plasmid PHP40099, **Table 2 for** the Genetic Elements in T-DNA Region of Plasmid PHP40099, and Figure 3 for the T-DNA region map from Plasmid PHP40099.

c. Molecular Characterisation

Molecular characterization of transgenic events determines the insertion copy number, insertion intactness, and the 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 intactness, 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.

Please refer to Appendix A for the complete sequence of the T-DNA region of PHP40099.

Southern-by-Sequencing (SbS) Analysis for Copy Number, Intactness, and Confirmation of the Absence of Vector Backbone Sequences

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 NGS procedure, and the results were analysed using bioinformatics tools.

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

Multiple sequence reads are generated 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 indicates the presence of rearrangements or additional insertions of plasmid 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 6**.

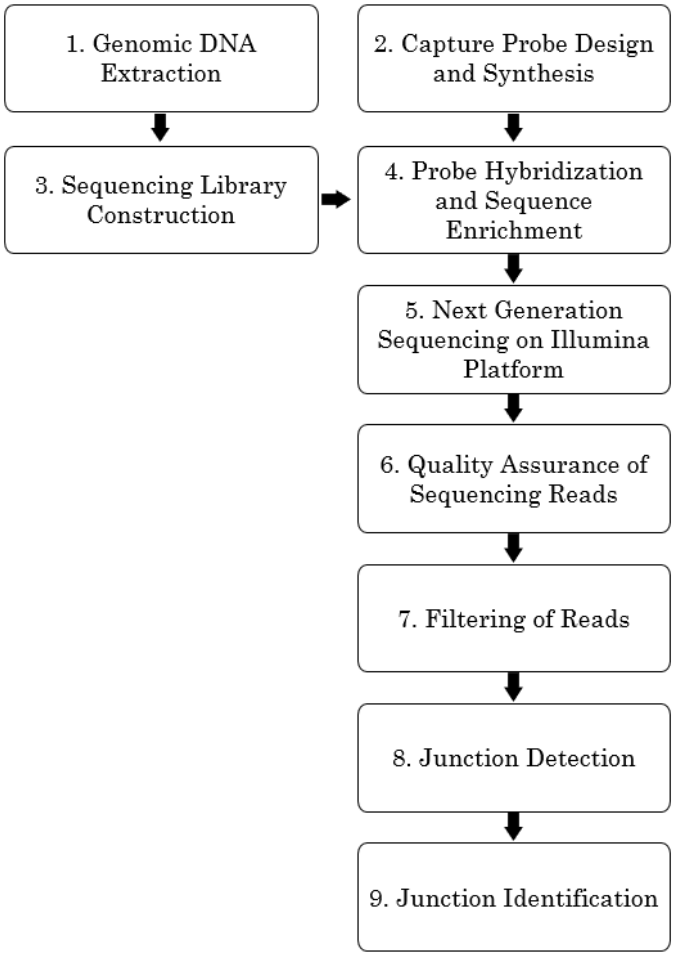


Figure 6. SbS Process Flow Diagram

SbS analysis was used to determine insertion copy number and intactness and confirm the absence of plasmid backbone sequences in DP202216 maize plants. SbS using full-coverage probes comprising the entire sequence of the PHP40099 transformation plasmid was conducted on eight plants from the T1 generation of DP202216 maize. The PH17AW maize (unmodified maize that has the same genetic background as DP202216 maize but does not contain the DP202216 insertion, referred to as control maize) and a positive control sample (control maize DNA spiked with PHP40099 plasmid DNA at a level corresponding to one copy of PHP40099 plasmid per copy of the maize genome) were also analysed by SbS.

The DP202216 T1 maize plants and control maize plants were germinated and analysed by polymerase chain reaction (PCR) to confirm the presence or absence of the *zmm28* and *mo-pat* genes (Kalla, C., and TeRonde, S.

2018). The eight DP202216 maize plants were also tested with an event-specific assay for the DP202216 insertion. Six DP202216 maize plants were positive for the *zmm28* and *mo-pat* genes and also confirmed to contain the inserted PHP40099 T-DNA by event-specific PCR analysis; the remaining two DP202216 maize plants were shown to be negative for the insertion (negative plants; **Table 3**). The control maize plant was negative for all PCR assays, indicating it did not contain the inserted DNA.

Following SbS analysis, each of the six plants that were determined to be positive for the DP202216 insertion resulted in the same two unique junctions that were consistent across all six plants. SbS analysis result for one representative plant is presented in **Figure 7**, and those for the other five plants that were positive for the DP202216 insertion are provided in **Appendix B**. The 5' junction for all six plants started with 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, indicating minor truncations of the T-DNA borders. Right Border and Left Border termini deletions often occur in *Agrobacterium*-mediated transformation (Kim *et al.*, 2007). These locations were identical across all six plants, indicating that the DP202216 DNA insertion is consistent and stable across the T1 generation of DP202216 maize. The number of sequence reads at the 5' and 3' junctions for each plant is provided in **Table 3**. There were no other junctions between the PHP40099 sequences and the maize genome in these six plants, indicating that there are no additional plasmid-derived insertions present in DP202216 maize. Additionally, there were no junctions between non-contiguous regions of the PHP40099 T-DNA identified, indicating that there are no rearrangements or additional truncations in the inserted DNA, other than the Right and Left Border truncations noted above. A schematic diagram of the DP202216 insertion was developed based on the SbS results and is provided in **Figure 8**.

Several genetic elements in the PHP40099 T-DNA (**Figure 3**) are derived from maize, and thus the homologous elements in the genome of the PH17AW control plant and plants lacking any insertion will be captured by the full-coverage probes used in the SbS analysis. These endogenous elements (*zm-gos2* and *ubi*ZM1 promoters, *ubi*ZM1 5'UTR, *ubi*ZM1 intron, and *zmm28*) will have sequencing reads in the SbS results for plants lacking any insertion due to the homologous elements in the PH17AW maize genome.

SbS results for the control maize plant and the positive control sample are presented in **Figures 9 and 10**, respectively. Sequencing reads were detected in the control maize (**Figure 9**); however, coverage above background level (35x) was obtained only for the endogenous genetic elements derived from the maize genome. These sequence reads were from capturing and sequencing of these genetic elements in their normal context within the PH17AW maize genome. Variation in coverage of the endogenous elements is due to sequence variation between the PH17AW 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 genome, 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 of the positive control sample resulted in sequence coverage across the entire length of the PHP40099 plasmid (**Figure 10**). This demonstrates that the SbS analysis utilizing the full-coverage probe library is sensitive enough to detect PHP40099 sequences at a concentration equivalent to one copy of PHP40099 per copy of the maize genome. Junctions were not detected between plasmid and genomic sequences, indicating that the sequence reads were due to the spiked-in plasmid, or to the endogenous maize genetic elements also detected in the control maize.

The two DP202216 maize plants that were confirmed to be negative for the insertion were also analysed by SbS, and results are shown in **Appendix B**. 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. There were no junctions between the PHP40099 sequences and the maize genome detected in these two plants, indicating that these plants did not contain any insertions derived from PHP40099.

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

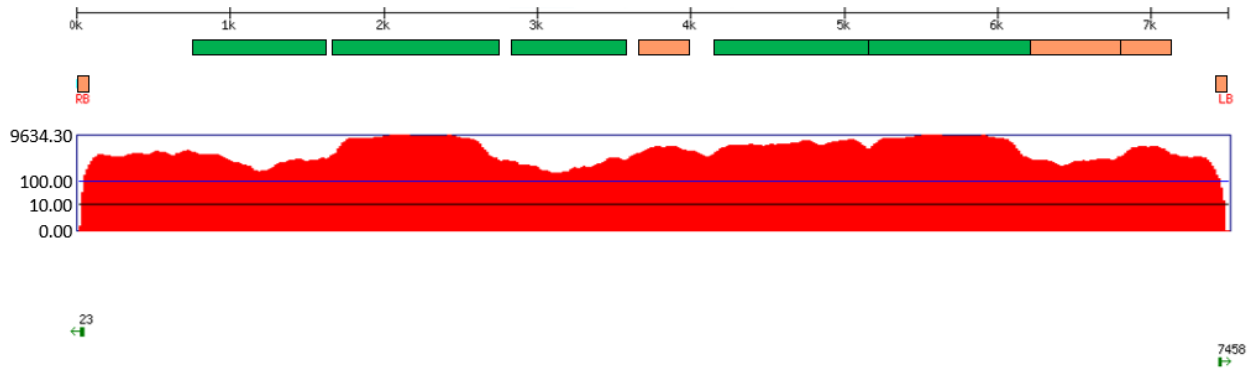
SbS analysis of the T1 generation of DP202216 maize demonstrated that there is a single, intact insertion derived from the PHP40099 T-DNA in DP202216 maize and that no additional insertions are present in its genome.

Table 3. PCR Analysis and SbS Junction Reads of DP202216 Maize Plants

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

1. The presence of the DP202216 DNA insertion is based on event-specific PCR results.
2. A total number of sequence reads across the 5' junction of the DP202216 insertion.
3. Unique sequence reads defining 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.
4. A total number of sequence reads across the 3' junction of the DP202216 insertion.
5. Unique sequence reads defining 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.

A.



B.

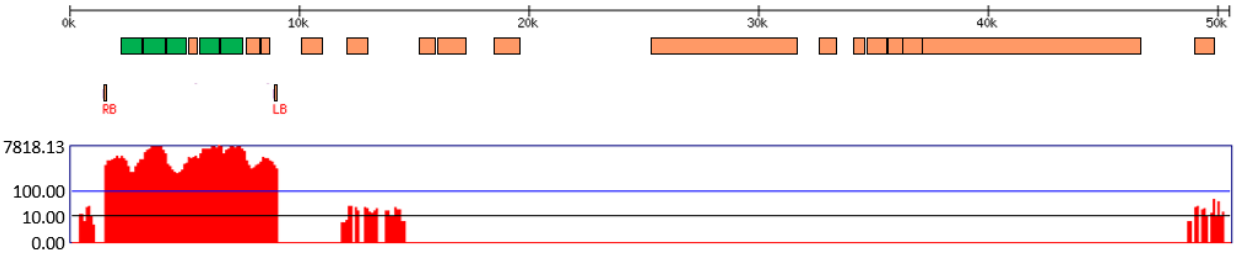


Figure 7. SbS Analysis for a Representative DP202216 Maize Plant

SbS results for a representative T1 generation DP202216 maize plant (ID 335728647 in **Table 3**) that was positive for the DP202216 insertion as confirmed by PCR. The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate genetic elements in plasmid PHP40099 derived from the maize genome, while tan bars indicate the genetic elements derived from other sources. **A)** SbS results aligned against the PHP40099 T-DNA (7,470 bp) intended for insertion. Green arrows indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the intact T-DNA sequence. The insertion comprises bp 23 to 7,458 of the PHP40099 T-DNA shown in **Figure 3**. 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 backbone sequence present in DP202216 maize.

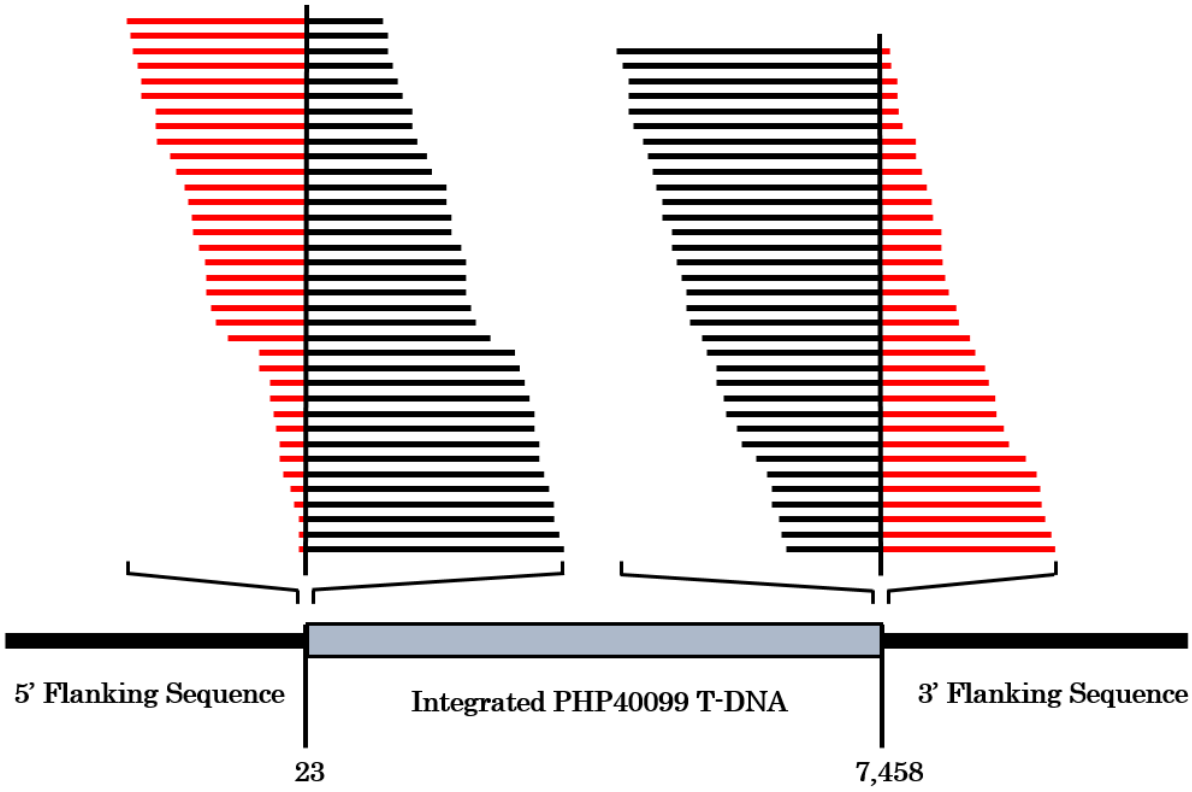
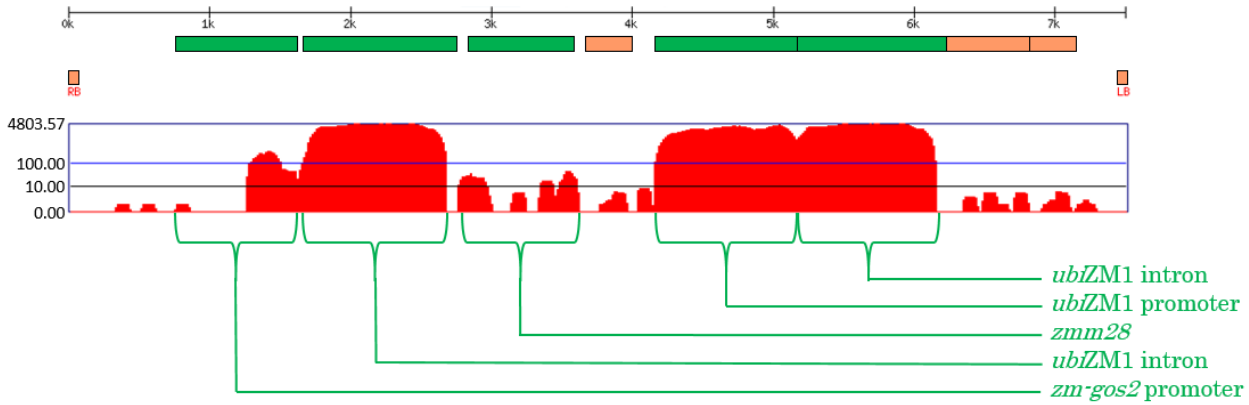


Figure 8. Map of the DP202216 Maize Insertion [redacted] 2019)

Schematic map of the PHP40099 T-DNA insertion in DP202216 maize based on the SbS analysis. The flanking maize genomic regions are indicated in the map. A single copy of the PHP40099 T-DNA, shown by the grey box, is integrated into the maize genome. Vertical lines show the locations of the two unique junctions. The numbers below the map indicate the bp location of the junction nucleotide in reference to the sequence of the PHP40099 T-DNA (**Table 2**). Representative individual sequencing reads across the junctions are shown as stacked lines above each junction (not to scale); red indicates genomic flanking sequence and black indicates T-DNA sequence within each individual read.

A.



B.

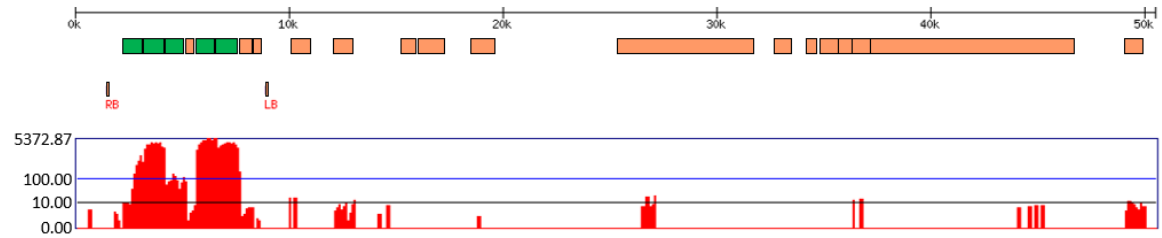
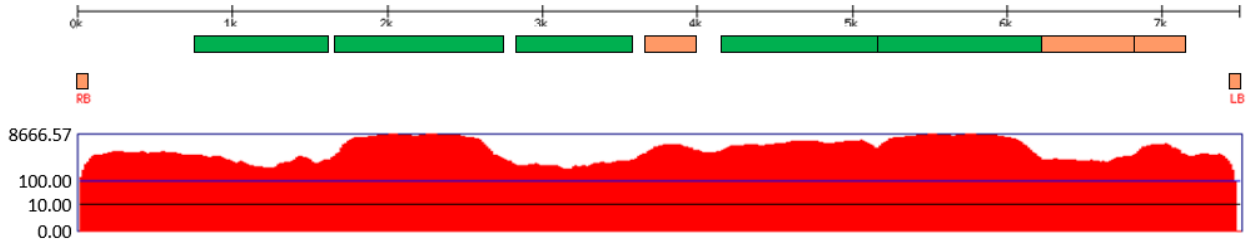


Figure 9. SbS Analysis for Control Maize

The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale with indications of number of reads. Green bars above the coverage graph indicate 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 (labelled in green font). Variation in coverage of the endogenous elements is due to sequence variations between the control maize and the maize varieties that the corresponding genetic elements in PHP40099 were derived from. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions in the control maize, and the sequence reads are solely due to the endogenous elements present in the PH17AW 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 plasmid backbone sequence present in the PH17AW control maize.

A.



B.

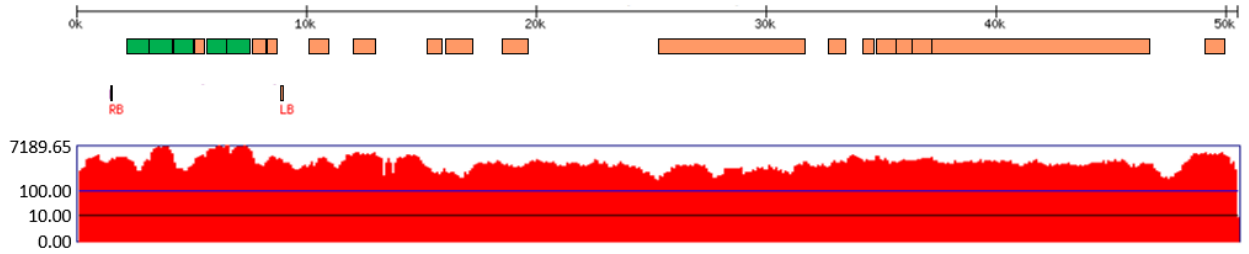


Figure 10. SbS Analysis for the Positive Control Sample

The positive control sample consisted of control maize 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 sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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.

Event-Specific Detection Method - Please refer to Attachment 2, Confidential Commercial Information.

d. Breeding Process

Please refer to Figure 4 for a schematic overview of the transformation and event development process for DP202216 maize. The subsequent breeding of DP202216 maize proceeded as indicated in Figure5 to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial lines.

e. Stability of Genetic Changes

Southern Analysis for Stable Genetic Inheritance [redacted], 2018)

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 T1, T2, BC1F1, BC3F3, and BC3F6 generations of DP202216 maize and control maize lines (PH17W and PHR1J) were analysed 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 analysed confirms that the DP202216 maize insertion is stable and equivalent across multiple generations. All probes and the restriction enzyme used for the analysis are indicated on the schematic maps of PHP40099 and the PHP40099 T-DNA region (**Figures 2 and 3**, respectively) and outlined in **Table 4**.

Restriction enzyme *Nco* I was selected to verify the stability of the DP202216 insertion because there is a single *Nco* I restriction site within the PHP40099 T-DNA region (**Figure 3**), which provides a means to uniquely identify the event, as additional sites would be in the adjacent flanking genomic DNA (**Figure 13**). Genomic DNA samples from five generations (T1, T2, BC1F1, BC3F3, and BC3F6) 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 genomic border regions of the DP202216 insertion were not changed 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.

Since the *zmm28* gene is derived from the maize genome, the *zmm28* gene probe is expected to hybridize to its endogenous gene and other genes with homologous sequence found in the maize genome, and thus additional hybridization bands in all the DP202216 and control maize samples were expected. The T1 and T2 generation DP202216 samples are of PH17AW control maize genetic background; whereas those of the BC1F1, BC3F3, and BC3F6 generations are of PHR1J control maize genetic background. Endogenous bands of DP202216 maize at the T1 and T2 generations aligned with the PH17AW control maize; whereas endogenous bands of DP202216 maize at BC1F1, BC3F3, and BC3F6 generations matched those in the PHR1J control maize line. These endogenous bands are indicated by asterisks (*) and grey shading in **Table 5**.

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 5, Figure 14**). 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 5, Figure 14**). 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 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 analysed (**Table 5, Figure 15**). 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 confirms 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 C.**

Table 4. 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	*zmm28*	901	2,664 to 3,564
2018-DP-0002	*mo-pat*	660	6,135 to 6,794

[1] The probe position is based on the PHP40099 T-DNA map (**Figure 12**).

Table 5. 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
zmm28	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*	14
mo-pat	12,605	>3,932	~7,000	15

An (*) and grey shading indicates the designated bands due to hybridization to endogenous sequences. These bands were identified in the maize control lines PH17AW and PHR1J that were analysed.

** Endogenous bands present in DP202216 maize of T1 & T2 generations and PH17AW control maize samples.

*** Endogenous bands present in DP202216 maize of BC1F1, BC3F3, and BC3F6 generations and PHR1J control maize samples.

[1] Observed fragment sizes are approximated from the DIG-labelled 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.

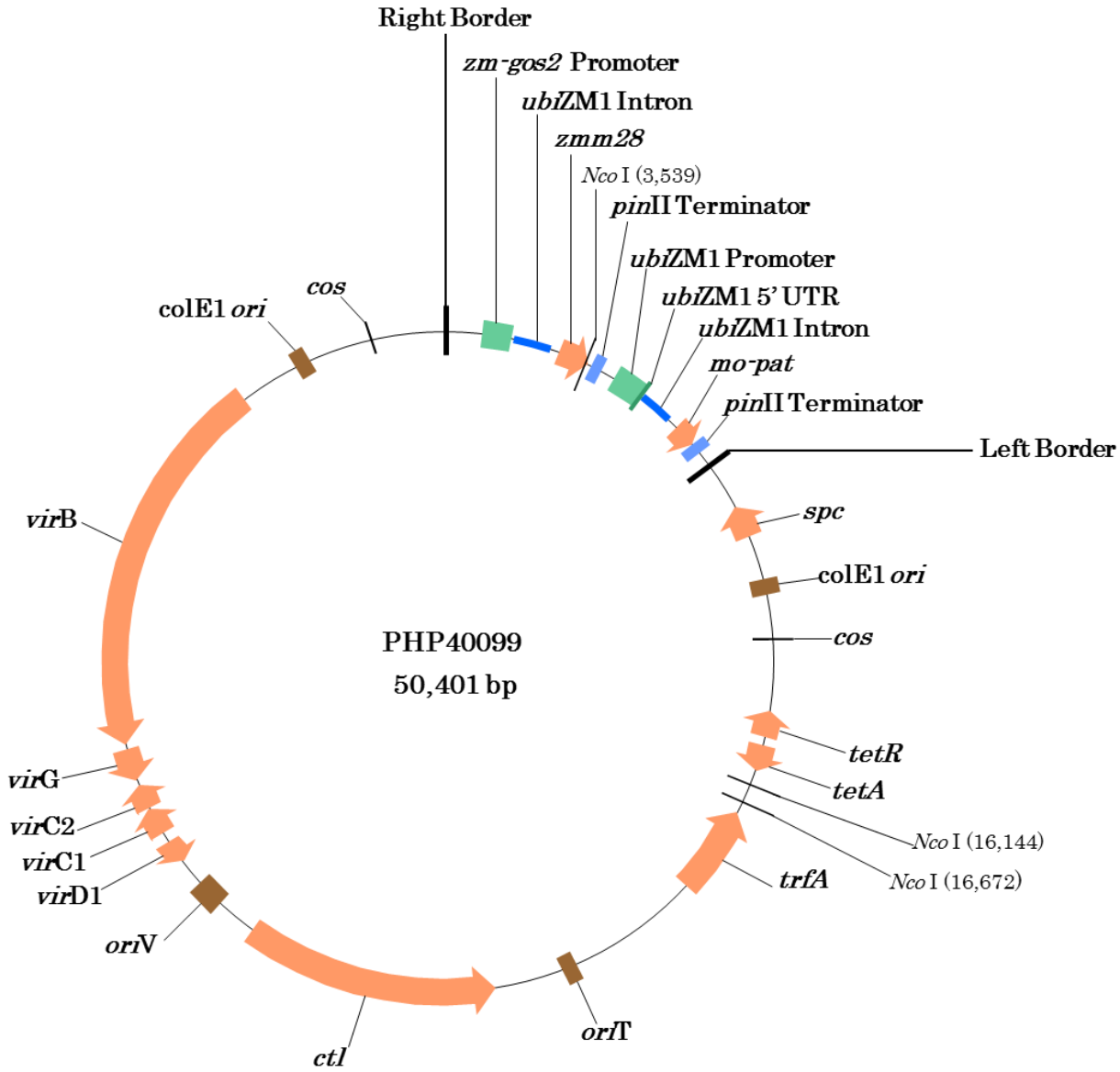
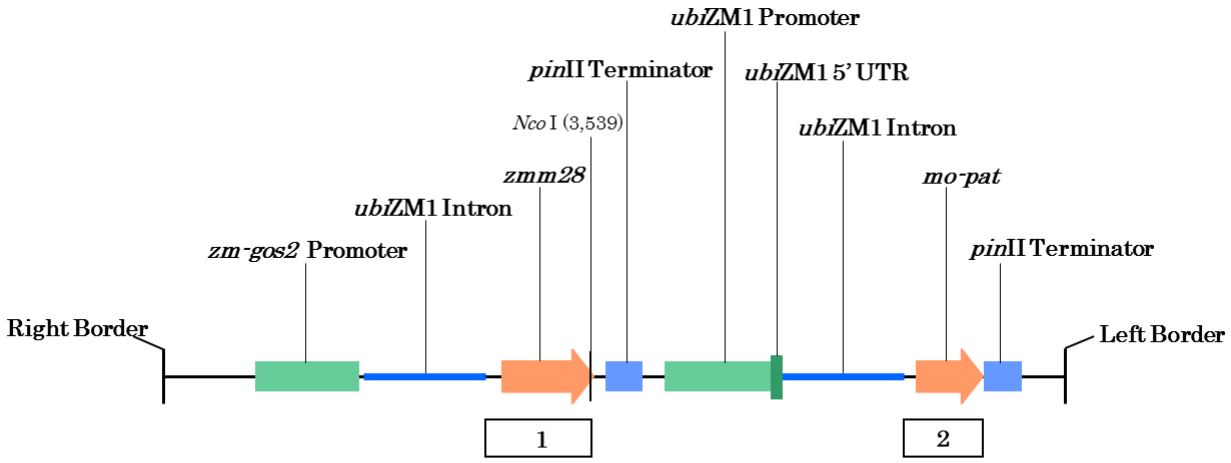


Figure 11. Map of Plasmid PHP40099

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 12**) that was transferred during *Agrobacterium*-mediated transformation. Plasmid size is 50,401 bp.



Number	Probe Name
1	*zmm28*
2	*mo-pat*

Figure 12. Map of Plasmid PHP40099 T-DNA

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.

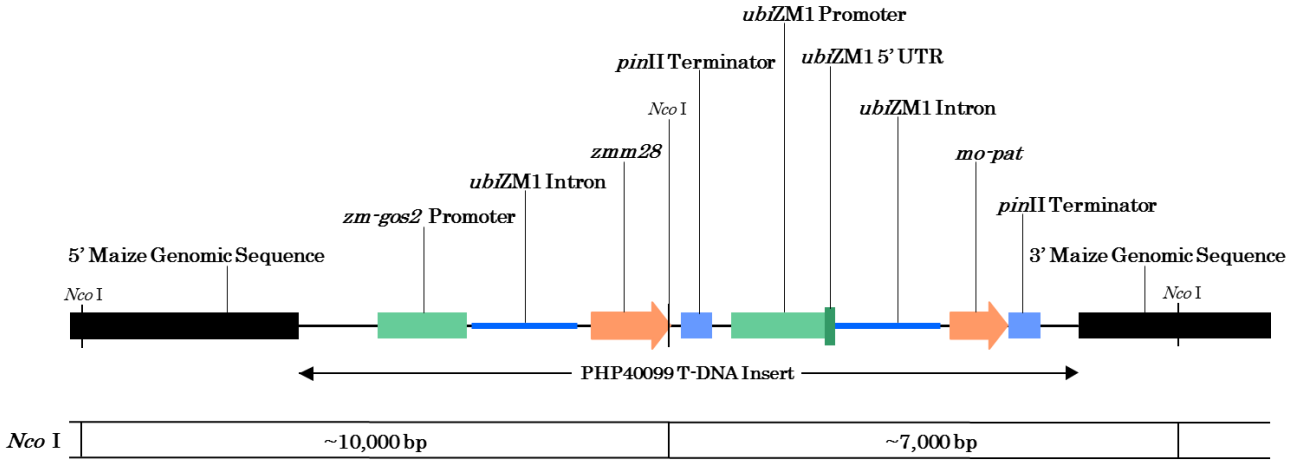
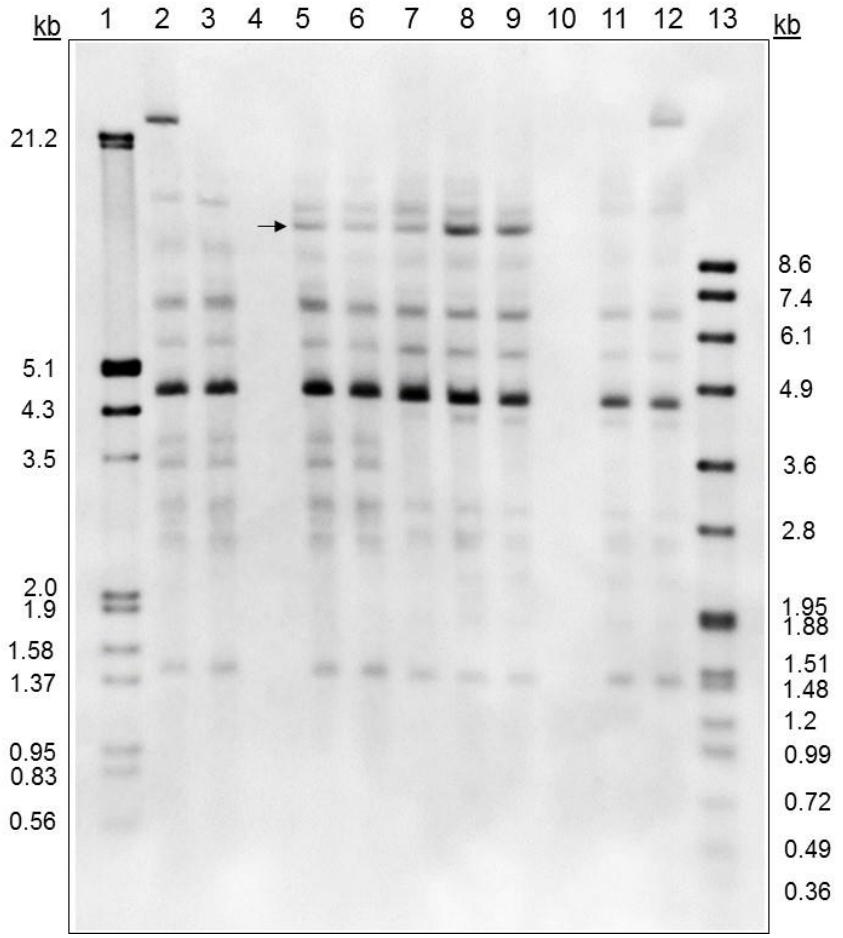


Figure 13. Map of the DP202216 Maize Insertion

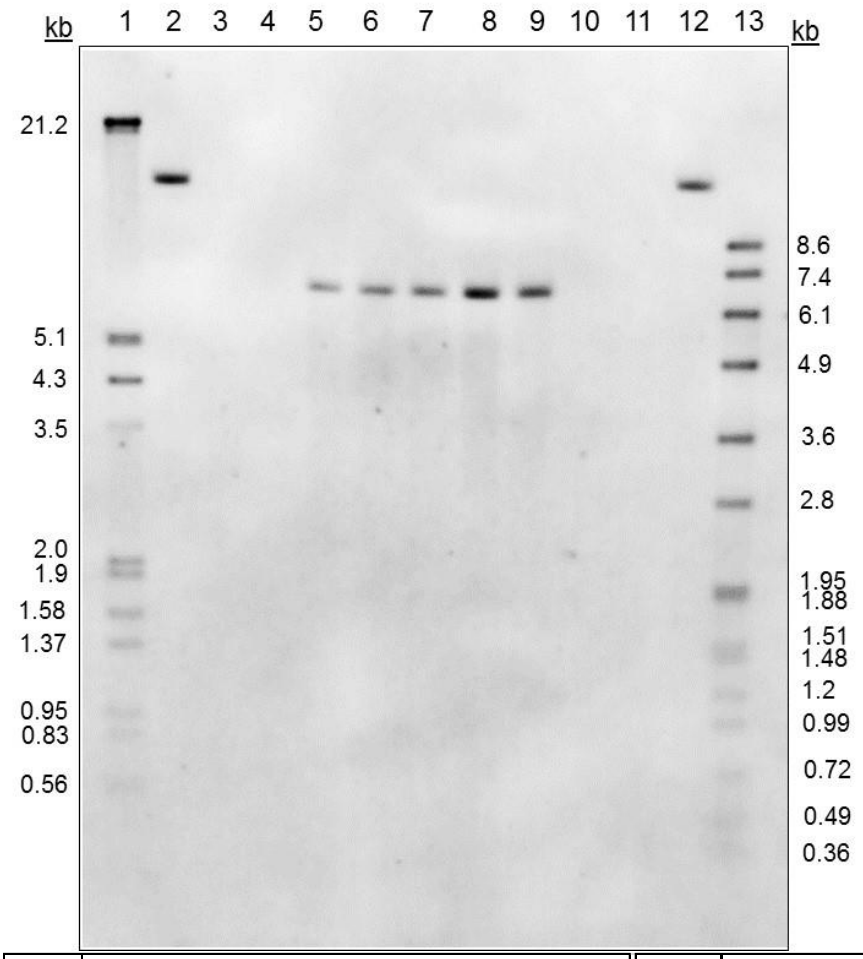
Map of the DP202216 maize insertion region including the *Nco* I restriction enzyme sites. The flanking maize genomic DNA is represented by the horizontal black rectangular bars. A single copy of the PHP40099 T-DNA integrated into the maize genome. *Nco* I restriction sites are indicated with the sizes 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 BC3F3 generation
2	1 copy PHP40099 + PH17AW control maize	9	DP202216 maize BC3F6 generation
3	PH17AW control maize	10	Blank
4	Blank	11	PHR1J control maize
5	DP202216 maize T1 generation	12	1 copy PHP40099 + PHR1J control maize
6	DP202216 maize T2 generation	13	DIG-labeled DNA marker VII
7	DP202216 maize BC1F1 generation		

Figure 14. Southern Blot Analysis of DP202216 Maize; Nco I Digest with *zmm28* Gene Probe

Genomic DNA isolated from leaf tissues of DP202216 maize from T1, T2, BC1F1, BC3F3, and BC3F6 generations, and PH17AW and PHR1J 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-labelled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).



Lane	Sample	Lane	Sample
1	DIG-labeled DNA marker III	8	DP202216 maize BC3F3 generation
2	1 copy PHP40099 + PH17AW control maize	9	DP202216 maize BC3F6 generation
3	PH17AW control maize	10	Blank
4	Blank	11	PHR1J control maize
5	DP202216 maize T1 generation	12	1 copy PHP40099 + PHR1J control maize
6	DP202216 maize T2 generation	13	DIG-labeled DNA marker VII
7	DP202216 maize BC1F1 generation		

Figure 15. Southern Blot Analysis of DP202216 Maize; Nco I Digest with *mo-pat* Gene Probe

Genomic DNA isolated from leaf tissues of DP202216 maize from T1, T2, BC1F1, BC3F3, and BC3F6 generations, and PH17AW and PHR1J 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-labelled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).

Mendelian Inheritance of the T-DNA Insert [redacted] 2018)

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 (T2, F1 [PH17AW/PHR1J], BC1F1, BC3F3, and BC3F6; **Figure 3**) 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 analysed for the presence of inserted DNA by event-specific PCR and for the presence of each of the introduced genes by gene-specific PCR. The herbicide tolerance 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 T2, and 1:1 for F1 (PH17AW/PHR1J) and BC1F1. All plants of the BC3F3 and BC3F6 generations of DP202216 maize were confirmed to be positive (*i.e.*, not segregating) as expected for a homozygous generation.

Table 6. Summary of Genotypic and Phenotypic Segregation Results of Five Generations of DP202216 Maize [redacted] 2018).

DP202216 Generation	Expected Segregation Ratio	Observed Segregation Values[a]			Statistical Analysis	
	(Positive:Negative)	Positive	Negative	Total	Chi-Square[b]	P-Value
T2	3:1	80	20	100	1.33	0.2482
F1 (PH17AW/PHR1J)	1:1	54	46	100	0.64	0.4237
BC1F1	1:1	42	58	100	2.56	0.1096
BC3F3	Homozygous	100	0	100	--	--
BC3F6	Homozygous	100	0	100	--	--

[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) tolerance analysis were conducted for each plant in each entry. All PCR results matched the corresponding herbicide tolerance result for each plant analysed.

[b] Degrees of freedom = 1.

In every case, a positive plant tested positive for the presence of the DP202216 maize insertion; the *zmm28* and *mo-pat* genes; and the herbicide tolerance phenotype, indicating that the inserted T-DNA and its included 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 T2, F1 (PH17AW/PHR1J), and BC1F1 generations of DP202216 maize (**Table 6**). A chi-square test was not performed for the BC3F3 and BC3F6 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 D.**

Nucleotide Sequencing of the Introduced DNA and Genomic Flanking Regions - - Please refer to Attachment 2, Confidential Commercial Information.

Molecular Characterization of DP202216 Maize Conclusion

The molecular characterization of the inserted DNA in DP202216 maize was performed using SbS analysis, Southern analyses, phenotypic segregation analyses, and bioinformatics analysis. Together, these studies demonstrate that the introduced genes were integrated at a single point of insertion, are stably inherited across multiple generations, and segregate according to Mendel's law of inheritance.

B. Characterization and safety assessment of the new substance

B.1 Characterization and safety assessment of new substances

A compositional equivalence assessment demonstrated that the nutrient composition of DP202216 maize grain is comparable to that of non-GM maize. Additionally, the *ZMM28* and PAT proteins have been assessed for safety.

ZMM28

The information and data provided herein have established that the *ZMM28* protein present in DP202216 maize is identical to the *ZMM28* protein found in conventional maize. This equivalency was established based on the following criteria:

- 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 of the *ZMM28* protein from DP202216 maize and the *ZMM28* protein from control maize.

The safety of the *ZMM28* protein was evaluated, based on:

1. The safety of the source of the *zmm28* gene
2. A history of exposure to transcription factors in food
3. Identical amino acid sequence of the native and introduced *ZMM28* proteins in DP202216 maize with the *ZMM28* protein in non-modified conventional maize;
4. Identical amino acid sequence of the *ZMM28* protein in DP202216 maize to the *ZMM28* protein in sweet corn;
5. Homology of the *ZMM28* protein in D202216 maize with proteins in other commonly consumed food crops; and
6. The presence of the *ZMM28* protein in conventional maize, including sweet corn.

DP202216 maize is as safe as non-GM maize varieties in food and feed. The increased and extended expression of the *ZMM28* protein in DP202216 maize is unlikely to present an increased risk for adverse health effects due to consumption (Anderson *et al.*, 2019). Further, additional hazard identification and characterization studies (including *in silico* toxicity assessment, *in silico* allergenicity assessment, heat liability, digestibility in simulated gastric fluid, and acute oral toxicity study), which are typically conducted to assess the safety of newly expressed proteins in GM crops without a history of safe use are not necessary to assess safety of the *ZMM28* protein in DP202216 maize.

PAT

DP202216 maize is identical to the corresponding protein found in a number of approved events across several different crops that are currently in commercial use. This equivalency was established based on the following criteria:

- The DNA insertion in DP202216 maize was sequenced and the translated amino acid sequence of the encoded PAT protein was determined. The translated amino acid sequence of PAT protein in the DP202216 insertion was compared and found to be identical to the amino acid sequence of PAT protein in previously authorized events.
- Western blot analysis confirmed the expected and equivalent size of the PAT protein from DP202216 maize and the PAT reference standard protein and the PAT protein in previously authorized 1507 and 59122 maize lines.

The PAT protein has been previously assessed for potential toxicity and potential allergenicty and numerous regulatory agencies, and determined to pose no significant risks to the environment, human, or animal health. In addition, there is a considerable body of public information supporting the safety of the PAT protein (Hérouet *et al.*, 2005).

a. Biochemical Function and Phenotypic Effects

Identity of the *ZMM28* Protein [redacted] 2019)

The *zmm28* gene, which encodes the *ZMM28* protein, is endogenous to maize. DP202216 maize contains a *zmm28* gene cassette with a constitutive maize *zm-gos*2 promotor, 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 16**).

```
A   1    MGRGPVQLRR IENKINRQVT FSKRRNGLLK KAHEISVLCD AEVALIVFST
B   1    MGRGPVQLRR IENKINRQVT FSKRRNGLLK KAHEISVLCD AEVALIVFST
C   1    MGRGPVQLRR IENKINRQVT FSKRRNGLLK KAHEISVLCD AEVALIVFST

A  51    KGKLYEYSSH SSMEGILERY QRYSFEERAV LNPSIEDQAN WGDEYVRLKS
B  51    KGKLYEYSSH SSMEGILERY QRYSFEERAV LNPSIEDQAN WGDEYVRLKS
C  51    KGKLYEYSSH SSMEGILERY QRYSFEERAV LNPSIEDQAN WGDEYVRLKS

A 101    KLDALQKSQR QLLGEQLSSL TIKELQQLEQ QLDSSLKHIR SRKNQLMFDS
B 101    KLDALQKSQR QLLGEQLSSL TIKELQQLEQ QLDSSLKHIR SRKNQLMFDS
C 101    KLDALQKSQR QLLGEQLSSL TIKELQQLEQ QLDSSLKHIR SRKNQLMFDS

A 151    ISALQKKEKA LTDQNGVLQK FMEAEKEKNK ALMNAQLREQ QNGASTSSPS
B 151    ISALQKKEKA LTDQNGVLQK FMEAEKEKNK ALMNAQLREQ QNGASTSSPS
C 151    ISALQKKEKA LTDQNGVLQK FMEAEKEKNK ALMNAQLREQ QNGASTSSPS

A 201    LSPPIVPDSM PTLNIGPCQH RGAAESESEP SPAPAQANRG NLPPWMLRTV
B 201    LSPPIVPDSM PTLNIGPCQH RGAAESESEP SPAPAQANRG NLPPWMLRTV
C 201    LSPPIVPDSM PTLNIGPCQH RGAAESESEP SPAPAQANRG NLPPWMLRTV

A 251    K*
B 251    K*
C 251    K*
```

Figure 16. Sequence Alignment of the Deduced Amino Acid Sequence of the *ZMM28* Protein [redacted] 2019)

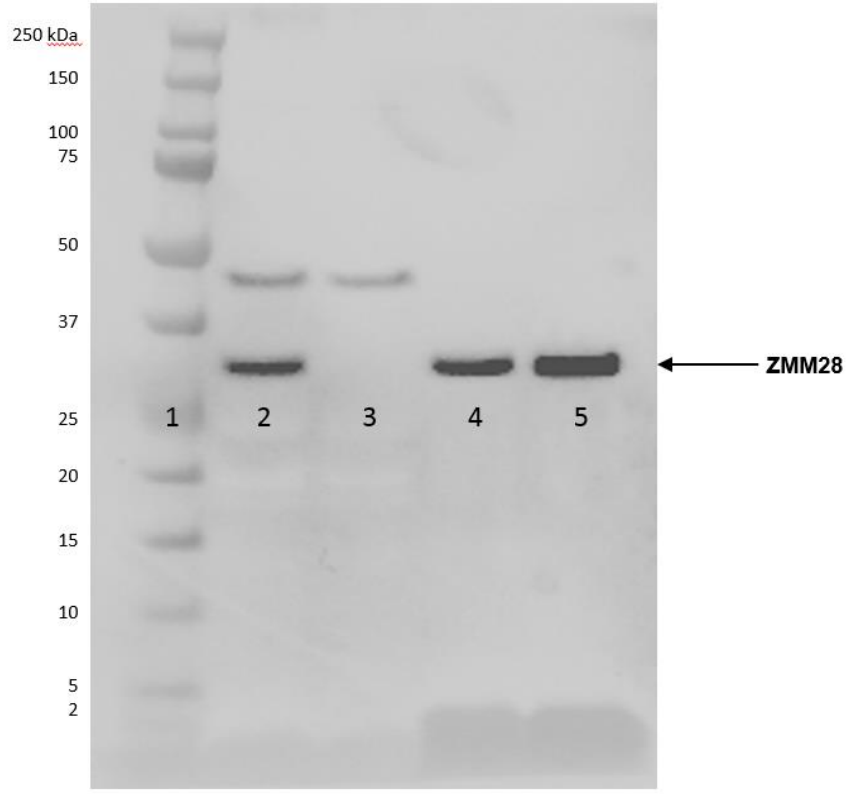
Deduced amino acid sequence alignment, where A represents the native *ZMM28* protein in DP202216, B represents introduced *ZMM28* protein in DP202216 maize, and C represents the *ZMM28* protein in the B73 reference genome (Genbank accession no: NP_001105155.1). The asterisk (*) indicates the translational stop codon.

Equivalence of the Native and Introduced *ZMM28* Protein in DP202216 Maize and Near-Isoline Control Maize ([redacted] 2018)

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

In the DP201226 maize R6 grain sample the *ZMM28* protein was detected on 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 (**Figure 17**, Lane 3). The 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. The protein detected in the DP202216 maize R6 grain, DP202216 maize V9 leaf, and control maize V9 leaf has the equivalent size (~28 kDa).

Western blot analysis demonstrated that the *ZMM28* protein in DP202216 maize and control 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 Analysis for the *ZMM28* Protein Derived from DP202216 Maize and Control Maize

Conclusion of Analysis of Amino Acid Sequence Alignment and Western Blot Analysis of the Native and Introduced *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. 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.

Mode of Action of *ZMM28* Protein

Based on *in silico* translation of the cDNA sequence in DP202216 maize, the introduced *zmm28* gene is identical to the native *zmm28* gene. Both the native and introduced *zmm28* genes encode the *ZMM28* protein, which is a MADS-box transcription factor. Based on physiological, biochemical, and molecular characterization, the increased and extended expression of the *ZMM28* protein in DP202216 maize enhances leaf source capacity, which results in plants with enhanced grain yield potential through improved plant vigour, increased photosynthetic capacity, and enhanced nutrient utilization (Wu, et.al., 2019).

Methods for Protein Characterisation and Equivalency Analyses

Please refer to **Appendix E.** for information pertaining to the methods used.

b. Evaluation of History of Safe Use of the *ZMM28* Protein in DP202216 Maize

Please refer to Section B.1 for the method by which the safety of the *ZMM28* protein was evaluated.

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

Transcription factors are present in commonly consumed foods and are a common component of human and animal diets (e.g., there are 1300 transcription factors identified in soybean, and over 2000 transcription factors in different varieties of rice (Parrott *et al.*, 2010). In general, since plants contain many different transcription factors, humans have a history of exposure to transcription factors in diet (Parrott *et al.*, 2010).

c. Unexpected Post-Translational Modification

As described in section B.B.1.a, the *zmm28* gene, which encodes the *ZMM28* protein, is endogenous to maize. 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. As shown in Figure 17, Western blot analysis demonstrated the ZMM28 protein in DP202216 maize and the ZMM28 protein in control maize have the expected and equivalent size (~28 kDa), indicating there is no unexpected post-translational modification.

d. Reading Frame Analysis [redacted] 2019)

All translated open reading frames (ORFs) of length ≥ 30 amino acids in the DP202216 maize (*Zea mays* L.) event sequence that are within the insertion or that cross the boundary between the insertion and its genomic borders were identified and evaluated.

Forty-five (45) ORFs ≥ 30 amino acids were identified for the DP202216 maize sequence.

The potential allergenicity of the translated ORFs was assessed by comparison of their sequences to the sequences in the Comprehensive Protein Allergen Resource (COMPARE) 2019 database (January 2019). The COMPARE database is compiled through a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins, and Bioinformatics (PATB) Committee. This database is peer-reviewed and contains 2,081 sequences. Two searches were performed to assess for potential allergenicity of the translated ORFs:

1. A search between the translated ORFs and protein sequences in the COMPARE database was conducted with FASTA using default parameters, except that the *E*-value was set to 0.0001. The returned alignments were inspected to identify any displaying ≥ 35% identity over an alignment length of ≥ 80 amino acids.
2. A search between the translated ORFs and protein sequences in the COMPARE database was conducted to identify any 8-contiguous amino acid matches to an allergen.

No alignments were returned between a translated ORF and any protein sequence in the COMPARE database. None of the translated ORFs in DP202216 maize produced an 8-contiguous amino acid match to an allergen. Collectively, these data indicate that there is no allergenicity concern regarding the translated ORFs in DP202216 maize.

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

No alignments were returned between a translated ORF and any protein sequence in the internal toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the translated ORFs.

Bioinformatics evaluation of the DP202216 insert did not generate any amino acid sequence similarities to known allergens, toxins, or other proteins that would be harmful to humans or animals.

B.2 New Proteins

a. Potential Toxicity and Allergenicity – *ZMM28*

The amino acid sequence alignment confirms that native and introduced *ZMM28* proteins from DP202216 maize are identical. The amino acid sequence alignment also confirms the introduced *ZMM28* protein in DP202216 maize is identical to the *ZMM28* protein from conventional maize (represented by the B73 reference genome; Genbank accession no: NP_001105155.1).

The amino acid sequence of the *ZMM28* protein in DP202216 maize is identical 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.*, 2019). 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 it demonstrates that the *ZMM28* protein is present in food.

DP202216 maize was genetically modified 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 **Table 8**). In R6 grain, the concentration of the *ZMM28* protein is within the range of the *ZMM28* protein concentrations detected in the R3 kernels from several sweet corn varieties (Anderson *et al.*, 2019). Collectively, the protein homology and protein concentration data demonstrate that the introduced *ZMM28* protein in DP202216 maize is equivalent to the *ZMM28* protein that is present in R3 kernels of selected varieties of sweet corn, which corresponds to the stage that sweet corn is typically consumed for food. Confirmation of the safety of source of the *zmm28* gene, the history of exposure to transcription factors in food, and the presence of the *ZMM28* protein in sweet corn supports, in part, the evaluation of history of safe use, which can be leveraged in the safety assessment of the *ZMM28* protein.

The increased and extended expression of the *ZMM28* protein in DP202216 maize is unlikely to present an increased risk for adverse health effects due to consumption (Anderson *et al.*, 2019). Further, additional hazard identification and characterization studies (including *in silico* toxicity assessment, *in silico* allergenicity assessment, heat liability, digestibility in simulated gastric fluid, and acute oral toxicity study), that are typically conducted to assess the safety of newly expressed proteins in GM crops without a history of safe use are not necessary to assess safety of the *ZMM28* protein in DP202216 maize.

b. Potential Toxicity and Allergenicity – PAT

Amino Acid Sequence of PAT Protein [redacted] 2018)

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

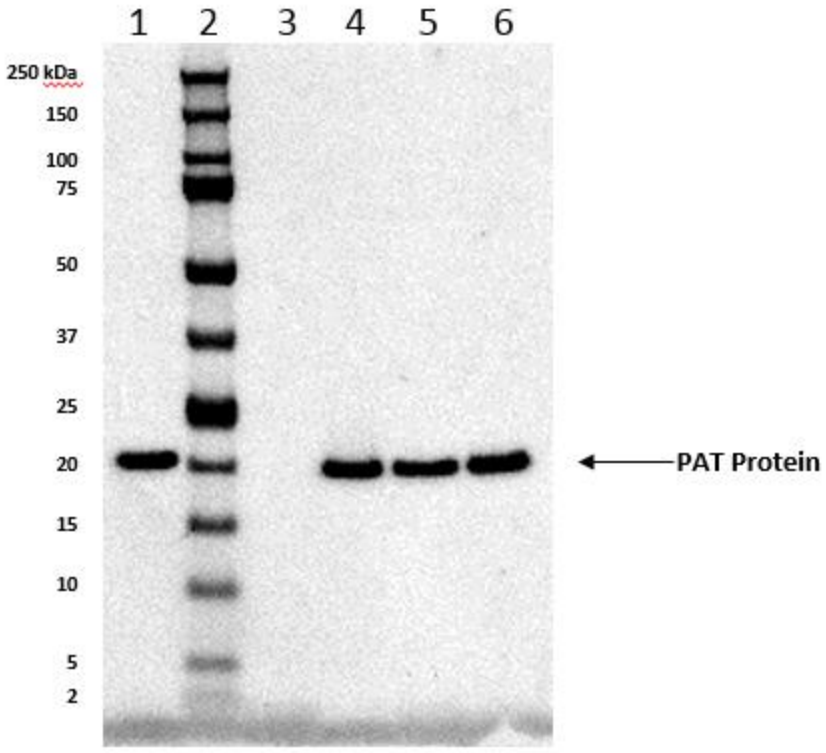
Figure 18. Deduced Amino Acid Sequence Alignment Between PAT Protein Encoded by pat Gene and PAT Protein encoded by *mo-pat* Gene [redacted] 2018)

Deduced amino acid sequence alignment, where PAT (*pat*) represents the deduced amino acid sequence from translation of the *pat* gene. PAT (*mo-pat*) represents the deduced amino acid sequence from translation of the *mo-pat* gene from DP202216 maize. The asterisk (*) indicates the translational stop codon.

As shown in **Figure 18**, the deduced amino acid sequence from translation of the *mo-pat* gene is identical to that of the already-deregulated PAT protein from translation of the *pat* gene, for which safety has been confirmed (Herouet *el al*., 2005) in a number of approved events across several different crops that are currently in commercial use.

Western Blot Analysis of PAT Protein Derived from DP202216 Maize [redacted] 2018).

Western blot analysis was conducted to confirm that the PAT protein from DP202216 maize has the expected and equivalent size (~21 kDa) as the PAT reference standard protein, and the PAT protein in previously approved events; 1507 (FSANZ Application A446) and 59122 (FSANZ Application A543).



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
5	1507 Maize-Derived PAT Protein
6	59122 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.

Figure 19. Western Blot Analysis of the PAT Protein Derived from Different Sources

Conclusion of Analysis of Amino Acid Sequence Alignment and Western Blot Analysis of the PAT Protein

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. Western blot results demonstrate the expected and equivalent size (~21 kDa) for the microbially derived PAT protein (**Figure 19**, Lane 1) and the DP202216 maize derived PAT protein (**Figure 19**, Lane 4). Additionally, western blot results demonstrate expected and equivalent size (~21 kDa) for the DP202216 maize derived PAT protein (**Figure 19**, Lane 4) and the 1507 and 59122 maize derived PAT proteins (**Figure 19**, Lanes 5 and 6, respectively). No PAT protein was detected from the near-isoline control maize (**Figure 19**, Lane 3).

Mode of Action of PAT Protein

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

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

Toxicity Assessment of the PAT Protein

***In silico* Toxicity Evaluation of PAT Protein [redacted] 2019)**

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

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 an internal 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 internal 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 internal 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 internal toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the PAT protein.

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 analysed 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 F**.

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 SGF containing pepsin (Hérouet *et al.*, 2005; OECD, 1999).

Acute Oral Toxicity Evaluation of PAT Protein [redacted] 2000)

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; [redacted] 2000), the dose was 5,000 mg PAT protein per kg body weight. During the two-week observation period, mortality and/or clinical or behavioural 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 course of 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 per kg body weight. Details regarding the materials and methods used for acute oral toxicity analysis are provided in **Appendix G**.

Allergenicity Assessment of the PAT Protein

***In silico* Allergenicity Evaluation of PAT Protein in DP202216 Maize [redacted] 2019)**

Assessing expressed proteins for potential cross-reactivity with known or putative allergens is a critical part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically-modified plant products (Codex Alimentarius Commission, 2003). In this study, a bioinformatic assessment of the PAT protein 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) 2019 database (January 2019) available at http://comparedatabase.org. This peer-reviewed database is compiled through a collaborative effort of the Health and Environmental Sciences Institute ((HESI) Protein Allergens, Toxins and Bioinformatics Committee (PATB) and is comprised of 2,081 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 an internally-produced Perl script 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.

Expression of *ZMM28* and PAT Proteins in DP202216 Maize [redacted] 2018)

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 commercial 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 modified (non-GM) 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 (**Table 7**) and processed as described in **Appendix H**. Time points for sampling were chosen to determine the range of protein concentrations throughout the growing season and for their relevance to commercial 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.

Table 7. 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.

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

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

Tissue	Growth Stage	ng *ZMM28*/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
		Mean	Range	Standard Deviation	Sample LLOQ	
	DP202216 Maize					
Leaf	V6	0.087[a]	<0.054 - 0.33	0.098[a]	0.054	10/24
	V9	0.28	0.066 - 0.72	0.18	0.054	0/24
	R1	0.32	0.084 - 0.66	0.15	0.054	0/24
	R4	0.12[a]	<0.054 - 0.22	0.049[a]	0.054	1/24
	R6	ND	<0.054	ND	0.054	24/24
Pollen	R1	0.015[a]	<0.028 - 0.028	0.0029[a]	0.028	23/24
Root	V9	0.031[a]	<0.027 - 0.078	0.018[a]	0.027	10/24
	R1	0.015[a]	<0.027 - 0.029	0.0041[a]	0.027	22/24
	R4	0.019[a]	<0.027 - 0.042	0.0091[a]	0.027	17/24
	R6	0.015[a]	<0.027 - 0.042	0.0058[a]	0.027	23/24
Forage	R4	0.049[a]	<0.036 - 0.12	0.020[a]	0.036	3/24
Whole Plant	V9	0.23	0.16 - 0.36	0.061	0.036	0/24
	R1	0.18	0.12 - 0.26	0.040	0.036	0/24
	R6	0.019[a]	<0.036 - 0.040	0.0045[a]	0.036	23/24
Grain	R6	0.012[a]	<0.0069 - 0.029	0.0070[a]	0.0069	6/24
	Control Maize					
Leaf	V6	0.062[a]	<0.054 - 0.28	0.081[a]	0.054	20/24
	V9	0.21	0.060 - 0.56	0.13	0.054	0/24
	R1	0.22[a]	<0.054 - 0.44	0.11[a]	0.054	2/24
	R4	0.079[a]	<0.054 - 0.14	0.037[a]	0.054	6/24
	R6	ND	<0.054	ND	0.054	24/24
Pollen	R1	ND	<0.028	ND	0.028	24/24
Root	V9	0.019[a]	<0.027 - 0.051	0.011[a]	0.027	18/24
	R1	0.016[a]	<0.027 - 0.042	0.0076[a]	0.027	21/24
	R4	ND	<0.027	ND	0.027	24/24
	R6	0.014[a]	<0.027 - 0.033	0.0040[a]	0.027	23/24
Forage	R4	0.029[a]	<0.036 - 0.058	0.013[a]	0.036	13/24
Whole Plant	V9	0.20	0.11 - 0.34	0.069	0.036	0/24
	R1	0.14	0.080 - 0.20	0.036	0.036	0/24
	R6	0.019[a]	<0.036 - 0.044	0.0053[a]	0.036	23/24
Grain	R6	ND	<0.0069	ND	0.0069	24/24

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. 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 and standard deviation.

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

Tissue	Growth Stage	ng PAT/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
		Mean	Range	Standard Deviation	Sample LLOQ	
	DP202216 Maize					
Leaf	V6	25	14 - 40	5.6	0.11	0/24
	V9	20	9.6 - 46	8.2	0.11	0/24
	R1	41	27 - 56	9.3	0.11	0/24
	R4	88	30 - 190	36	0.11	0/24
	R6	<0.11	<0.11	ND	0.11	24/24
Pollen	R1	76	66 - 110	10	0.22	0/24
Root	V9	17	0.072 - 30	9.2	0.054	0/24
	R1	7.4	2.7 - 15	3.8	0.054	0/24
	R4	11	4.5 - 20	4.1	0.054	0/24
	R6	11[a]	<0.054 - 23	7.1[a]	0.054	3/24
Forage	R4	32	16 - 48	8.1	0.036	0/24
Whole Plant	V9	32	20 - 46	6.8	0.036	0/24
	R1	26	15 - 36	5.1	0.036	0/24
	R6	21	0.52 - 68	16	0.036	0/24
Grain	R6	15	7.5 - 21	3.2	0.054	0/24

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.

Expressed Trait Protein Conclusion

Amino acid sequence analyses confirmed the native *ZMM28* protein and the introduced *ZMM28* protein in DP202216 maize are identical to each other. Western blot analysis confirmed that the introduced *ZMM28* protein in DP202216 maize and the *ZMM28* protein from near-isoline control maize have equivalent size. DP202216 maize expresses more *ZMM28* protein in tissues; however, the concentrations remain in the part per billion range.

The DP202216 maize-derived PAT protein and the PAT protein present in previously authorized events have the same amino acid sequence. Western blot analysis confirmed the expected and equivalent size (~21 kDa) for the DP202216 maize-derived PAT protein and 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).

B.5 Compositional analyses of the food produced

An assessment of the compositional equivalence of a GM product compared to that of a non-GM comparator with a history of safe use in feed is a critical part of the weight-of-evidence approach used to evaluate the safety of genetically modified plant products (Codex Alimentarius Commission, 2008; OECD, 1993). Compositional assessments of DP202216 maize were evaluated in comparison to concurrently grown non-GM, near-isoline maize (referred to as control maize) to identify statistical differences, and subsequently were evaluated in the context of normal ranges of variation established from multiple sources of non-GM, commercial maize data [redacted] 2018).

Nutrient composition analysis of DP202216 maize included proximates, fibre, 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).

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 commercial 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 non-GM commercial maize reference lines. Forage at R4 and grain at R6 growth stages were collected and analyzed for key nutritional components. A description of maize growth stages is provided in **Table 7**. All samples were collected from impartially selected, healthy, representative plants. Sample collection and processing methods are provided in **Appendix I.**

Determination of Nutrient Composition Analyte Concentrations

The collected forage and grain samples 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 U.S. 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 I.**

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.

To determine if any observed statistical differences were beyond the range of natural variation, statistical tolerance intervals were established from historical data of non-transgenic commercial maize lines. These commercial lines are typical of those grown in maize-growing regions; represent a wide range of varieties that would normally be planted commercially; and represent the normal range of variation of the maize crop. In addition, publicly available information was gathered on the range of natural variation of maize analyte concentrations (*i.e.,* literature range). Ranges of reference line analyte concentrations observed in this study were also reported in order to represent the specific environmental conditions in this study that may not have been included in the historical or literature data.

If the measured values of DP202216 maize fell within the statistical tolerance interval, literature range, or in-study reference range, then these measured values would be considered comparable to conventional maize.

The outcome of the nutrient composition assessment is provided in **Table 10**. Nutrient composition analysis results are provided in **Tables 11-21**. Details regarding statistical analysis methods are provided in **Appendix I.**

Table 10. Outcome of Nutrient Composition Assessment for DP202216 Maize

Subgroup	No Statistical Difference Identified	Statistical Difference Identified					Not Included in Statistical Analysis (All Data Values Below the Lower Limit of Quantification)
		All Data Values Within Tolerance Interval	One or More Data Values Outside Tolerance Interval, or Tolerance Interval Not Available			Adjusted P-Value<0.05	
			All Data Values Within Literature Range	One or More Data Values Outside Literature Range, or Literature Range Not Available			
				All Data Values Within Reference Data Range	One or More Data Values Outside Reference Data Range, or Reference Data Range Not Available		
Forage (R4 Growth Stage)							
Proximates, Fibre, and Mineral Composition	Crude Protein Crude Fat Crude Fibre ADF NDF Ash Carbohydrates Calcium Phosphorus	--	--	--	--	--	--
Grain (R6 Growth Stage)							
Proximates and Fibre Composition	Total Dietary Fibre Crude Protein Crude Fat Crude Fibre ADF NDF Ash Carbohydrates	--	--	--	--	--	--
Fatty Acid Composition	Palmitic Acid (C16:0)	--	--	--	--	--	Lauric Acid (C12:0)

Table 10. Outcome of Nutrient Composition Assessment for DP202216 Maize (continued)

Subgroup	No Statistical Difference Identified	Statistical Difference Identified					Not Included in Statistical Analysis (All Data Values Below the Lower Limit of Quantification)
		All Data Values Within Tolerance Interval	One or More Data Values Outside Tolerance Interval, or Tolerance Interval Not Available			Adjusted P-Value<0.05	
			All Data Values Within Literature Range	One or More Data Values Outside Literature Range, or Literature Range Not Available			
				All Data Values Within Reference Data Range	One or More Data Values Outside Reference Data Range, or Reference Data Range Not Available		
Grain (R6 Growth Stage)							
Fatty Acid Composition	Palmitoleic Acid (C16:1) Stearic Acid (C18:0) Oleic Acid (C18:1) Linoleic Acid (C18:2) Alpha-Linolenic Acid (C18:3) Arachidic Acid (C20:0) Eicosenoic Acid (C20:1) Behenic Acid (C22:0) Lignoceric Acid (C24:0)	--	--	--	--	--	Myristic Acid (C14:0) Heptadecanoic Acid (C17:0) Heptadecenoic Acid (C17:1) Eicosadienoic Acid (C20:2) Erucic Acid (C22:1)
Amino Acid Composition	Alanine Arginine Aspartic Acid Cystine Glutamic Acid Histidine Isoleucine Leucine Lysine	Glycine Methionine Serine	--	--	--	--	--

Table 10. Outcome of Nutrient Composition Assessment for DP202216 Maize (continued)

Subgroup	No Statistical Difference Identified	Statistical Difference Identified					Not Included in Statistical Analysis (All Data Values Below the Lower Limit of Quantification)
		All Data Values Within Tolerance Interval	One or More Data Values Outside Tolerance Interval, or Tolerance Interval Not Available			Adjusted P-Value<0.05	
			All Data Values Within Literature Range	One or More Data Values Outside Literature Range, or Literature Range Not Available			
				All Data Values Within Reference Data Range	One or More Data Values Outside Reference Data Range, or Reference Data Range Not Available		
Grain (R6 Growth Stage)							
Amino Acid Composition	Phenylalanine Proline Threonine Tryptophan Tyrosine Valine		--	--	--	--	--
Mineral Composition	Calcium Copper Iron Magnesium Manganese Phosphorus Potassium Sodium Zinc	--	--	--	--	--	--
Vitamin Composition	β-Carotene Vitamin B5 (Pantothenic Acid) Vitamin B6 (Pyridoxine)	Vitamin B1 (Thiamine) Vitamin B3 (Niacin)	--	--	--	--	Vitamin B2 (Riboflavin) β-Tocopherol

Table 10. Outcome of Nutrient Composition Assessment for DP202216 Maize (continued)

Subgroup	No Statistical Difference Identified	Statistical Difference Identified					Not Included in Statistical Analysis (All Data Values Below the Lower Limit of Quantification)
		All Data Values Within Tolerance Interval	One or More Data Values Outside Tolerance Interval, or Tolerance Interval Not Available			Adjusted P-Value<0.05	
			All Data Values Within Literature Range	One or More Data Values Outside Literature Range, or Literature Range Not Available			
				All Data Values Within Reference Data Range	One or More Data Values Outside Reference Data Range, or Reference Data Range Not Available		
Grain (R6 Growth Stage)							
Vitamin Composition	Vitamin B9 (Folic Acid) α-Tocopherol γ-Tocopherol δ-Tocopherol Total Tocopherols		--	--	--	--	--
Secondary Metabolite and Anti-Nutrient Composition	*p*-Coumaric Acid Ferulic Acid Inositol Phytic Acid Raffinose Trypsin Inhibitor	--	--	--	--	--	Furfural

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

Proximates, Fibre, and Minerals in DP202216 Maize Forage

Proximates, fibre, and minerals were analyzed in forage derived from DP202216 maize and 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, fibre, and minerals in maize forage demonstrate that DP202216 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 11. Proximates, Fibre, and Minerals Results for DP202216 Maize in Forage

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Crude Protein	Mean	8.32	8.41	4.30 - 12.6	3.14 - 16.32	5.80 - 11.8
	Range	6.30 - 11.2	6.20 - 10.8			
	Confidence Interval	7.59 - 9.05	7.68 - 9.15			
	Adjusted P-Value	--	0.830			
	P-Value	--	0.586			
Crude Fat	Mean	3.86	4.10	1.04 - 5.46	ND - 6.755	2.00 - 5.91
	Range	2.29 - 5.19	1.99 - 6.18			
	Confidence Interval	3.44 - 4.27	3.68 - 4.51			
	Adjusted P-Value	--	0.517			
	P-Value	--	0.239			
Crude Fibre	Mean	20.0	19.8	14.3 - 31.0	12.5 - 42	13.2 - 26.8
	Range	14.4 - 27.5	15.4 - 26.7			
	Confidence Interval	18.4 - 21.5	18.3 - 21.4			
	Adjusted P-Value	--	0.942			
	P-Value	--	0.873			
ADF	Mean	25.9	25.9	18.7 - 39.6	9.90 - 47.39	16.4 - 36.1
	Range	17.2 - 36.2	18.3 - 35.5			
	Confidence Interval	23.4 - 28.4	23.4 - 28.4			
	Adjusted P-Value	--	0.993			
	P-Value	--	0.981			
NDF	Mean	40.9	41.5	34.0 - 62.6	20.29 - 67.80	26.1 - 54.6
	Range	30.7 - 53.8	28.5 - 52.7			
	Confidence Interval	37.7 - 44.2	38.3 - 44.7			
	Adjusted P-Value	--	0.875			
	P-Value	--	0.707			
Ash	Mean	4.31	4.30	2.66 - 10.0	0.66 - 13.20	1.86 - 8.88
	Range	2.09 - 6.64	1.15 - 8.20			
	Confidence Interval	3.34 - 5.27	3.33 - 5.27			
	Adjusted P-Value	--	0.993			
	P-Value	--	0.974			
Carbohydrates	Mean	83.6	83.1	76.5 - 89.5	73.3 - 92.9	77.4 - 88.9
	Range	79.3 - 88.5	77.9 - 87.7			
	Confidence Interval	81.9 - 85.3	81.4 - 84.7			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.184			

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Calcium	Mean	0.210	0.216	0.0931 - 0.537	0.06 - 0.58	0.119 - 0.400
	Range	0.0777 - 0.315	0.157 - 0.398			
	Confidence Interval	0.178 - 0.243	0.184 - 0.249			
	Adjusted P-Value	--	0.815			
	P-Value	--	0.503			
Phosphorus	Mean	0.253	0.257	0.0956 - 0.454	0.07 - 0.55	0.109 - 0.344
	Range	0.149 - 0.349	0.125 - 0.347			
	Confidence Interval	0.216 - 0.291	0.220 - 0.295			
	Adjusted P-Value	--	0.830			
	P-Value	--	0.582			

Note: Proximates, fibre, 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.

Proximates and Fibre in DP202216 Maize Grain

Proximates and fibre were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in **Table 12**. No statistically significant differences (P-value < 0.05) were observed between DP202216 maize and control maize. The results of the analysis of proximates and fibre in maize grain demonstrate that DP202216 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 12. Proximates and Fibre Results for DP202216 Maize in Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Total Dietary Fibre	Mean	8.88	8.94	5.91 - 15.8	6.68 - 35.31	6.53 - 15.2
	Range	6.81 - 12.7	6.96 - 13.2			
	Confidence Interval	8.10 - 9.67	8.16 - 9.73			
	Adjusted P-Value	--	0.942			
	P-Value	--	0.879			
Crude Protein	Mean	8.36	8.58	7.18 - 13.2	5.72 - 17.26	7.12 - 11.7
	Range	7.08 - 10.5	7.02 - 10.6			
	Confidence Interval	7.78 - 8.93	8.01 - 9.16			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0670			
Crude Fat	Mean	4.19	4.21	2.58 - 6.00	1.363 - 7.830	2.45 - 5.86
	Range	3.09 - 5.36	3.10 - 5.35			
	Confidence Interval	3.93 - 4.46	3.95 - 4.48			
	Adjusted P-Value	--	0.942			
	P-Value	--	0.887			

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Crude Fibre	Mean	2.36	2.39	1.44 - 3.48	0.49 - 5.5	1.18 - 4.04
	Range	1.71 - 3.14	1.13 - 3.06			
	Confidence Interval	2.19 - 2.52	2.23 - 2.55			
	Adjusted P-Value	--	0.849			
	P-Value	--	0.649			
ADF	Mean	4.24	4.55	2.64 - 6.26	1.41 - 11.34	2.89 - 7.94
	Range	3.45 - 5.77	2.87 - 6.88			
	Confidence Interval	3.97 - 4.52	4.27 - 4.82			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.118			
NDF	Mean	9.74	9.48	7.22 - 20.8	4.28 - 22.64	5.87 - 12.7
	Range	6.88 - 11.4	6.86 - 11.3			
	Confidence Interval	9.26 - 10.2	9.00 - 9.96			
	Adjusted P-Value	--	0.545			
	P-Value	--	0.273			
Ash	Mean	1.27	1.30	0.976 - 1.80	0.616 - 6.282	0.830 - 1.63
	Range	0.810 - 1.43	0.952 - 1.54			
	Confidence Interval	1.15 - 1.39	1.17 - 1.42			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.112			
Carbohydrates	Mean	86.1	85.9	80.2 - 88.0	77.4 - 89.7	81.5 - 88.1
	Range	83.6 - 88.0	83.9 - 88.5			
	Confidence Interval	85.4 - 86.9	85.1 - 86.6			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.130			

Note: Proximates and fibre unit of measure is % dry weight.

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 13** and **14**. 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-GM near-isoline control maize and non-GM commercial maize.

Table 13. Fatty Acid Results for DP202216 Maize in Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Lauric Acid (C12:0)	Mean	<LLOQ[a]	<LLOQ[a]	0.00 - 0.209[b]	ND - 0.698	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Myristic Acid (C14:0)	Mean	<LLOQ[a]	<LLOQ[a]	0.00 - 0.267[b]	ND - 0.288	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Palmitic Acid (C16:0)	Mean	10.6	10.6	9.23 - 26.0	6.81 - 39.0	10.0 - 14.2
	Range	10.3 - 11.7	10.3 - 11.3			
	Confidence Interval	10.4 - 10.9	10.4 - 10.8			
	Adjusted P-Value	--	0.867			
	P-Value	--	0.688			
Palmitoleic Acid (C16:1)	Mean	0.0775	0.0787	0 - 0.463	ND - 0.67	0.0349 - 0.136
	Range	0.0369 - 0.105	0.0385 - 0.107			
	Confidence Interval	0.0643 - 0.0906	0.0655 - 0.0919			
	Adjusted P-Value	--	0.836			
	P-Value	--	0.627			
Heptadecanoic Acid (C17:0)	Mean	<LLOQ[a]	<LLOQ[a]	0 - 0.245	ND - 0.203	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Heptadecenoic Acid (C17:1)	Mean	<LLOQ[a]	<LLOQ[a]	0.00 - 0.135[b]	ND - 0.131	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			

Stearic Acid (C18:0)	Mean	2.06	2.09	1.31 - 3.94	ND - 4.9	1.39 - 2.54
	Range	1.77 - 2.40	1.66 - 2.42			
	Confidence Interval	1.91 - 2.22	1.94 - 2.24			
	Adjusted P-Value	--	0.545			
	P-Value	--	0.265			
Oleic Acid (C18:1)	Mean	29.9	29.9	18.9 - 39.4	16.38 - 42.81	22.4 - 34.3
	Range	28.3 - 32.3	27.5 - 32.5			
	Confidence Interval	28.8 - 30.9	28.9 - 30.9			
	Adjusted P-Value	--	0.878			
	P-Value	--	0.765			
Linoleic Acid (C18:2)	Mean	55.0	54.9	28.9 - 64.4	13.1 - 67.68	45.5 - 60.6
	Range	51.3 - 56.7	51.2 - 57.3			
	Confidence Interval	53.6 - 56.4	53.5 - 56.3			
	Adjusted P-Value	--	0.830			
	P-Value	--	0.571			
Alpha-Linolenic Acid (C18:3)	Mean	1.33	1.33	0.0362 - 2.15	ND - 2.33	0.922 - 2.21
	Range	1.20 - 1.53	1.16 - 1.56			
	Confidence Interval	1.26 - 1.40	1.26 - 1.40			
	Adjusted P-Value	--	0.944			
	P-Value	--	0.902			
Arachidic Acid (C20:0)	Mean	0.388	0.390	0.296 - 0.916	0.267 - 1.2	0.296 - 0.558
	Range	0.337 - 0.498	0.344 - 0.526			
	Confidence Interval	0.353 - 0.424	0.354 - 0.426			
	Adjusted P-Value	--	0.830			
	P-Value	--	0.576			
Eicosenoic Acid (C20:1)	Mean	0.256	0.258	0.0380 - 0.693	ND - 1.952	0.224 - 0.521
	Range	0.234 - 0.290	0.236 - 0.304			
	Confidence Interval	0.243 - 0.270	0.245 - 0.271			
	Adjusted P-Value	--	0.799			
	P-Value	--	0.470			
Eicosadienoic Acid (C20:2)	Mean	<LLOQ[a]	<LLOQ[a]	0.00 - 0.825[b]	ND - 2.551	<LLOQ[a]
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			

Behenic Acid (C22:0)	Mean	0.0873	0.0871	0 - 0.453	ND - 0.5	0.0691 - 0.314
	Range	0.0700 - 0.182	0.0710 - 0.204			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Lignoceric Acid (C24:0)	Mean	0.165	0.167	0 - 0.639	ND - 0.91	0.0796 - 0.391
	Range	0.0708 - 0.258	0.0712 - 0.283			
	Confidence Interval	0.0729 - 0.204	0.0823 - 0.206			
	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 analysed because all sample values in the current study and in historical commercial reference lines were below the lower limit of quantification (LLOQ). This analyte was excluded from the report table.
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] A 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 14. Number of Fatty Acid Sample Values Below the Lower Limit of Quantification for DP202216 Maize in 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 analysed because all sample values in the current study and in historical commercial reference lines were below the lower limit of quantification (LLOQ). This analyte was excluded from the report table.
[a] This analyte had <50% below-LLOQ sample values in DP202216 maize and the control maize, and was subjected to the mixed model analyses.

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 15**.

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 of 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-GM near-isoline control maize and non-GM commercial maize.

Table 15. Amino Acid Results for DP202216 Maize in Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Alanine	Mean	0.609	0.623	0.492 - 1.08	0.44 - 1.48	0.500 - 0.937
	Range	0.503 - 0.803	0.479 - 0.800			
	Confidence Interval	0.554 - 0.663	0.569 - 0.677			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.103			
Arginine	Mean	0.380	0.390	0.317 - 0.568	0.12 - 0.71	0.305 - 0.502
	Range	0.309 - 0.429	0.315 - 0.450			
	Confidence Interval	0.356 - 0.405	0.365 - 0.414			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0825			
Aspartic Acid	Mean	0.530	0.540	0.445 - 0.916	0.33 - 1.21	0.429 - 0.779
	Range	0.434 - 0.649	0.412 - 0.651			
	Confidence Interval	0.488 - 0.572	0.498 - 0.582			
	Adjusted P-Value	--	0.517			
	P-Value	--	0.243			
Cystine	Mean	0.191	0.201	0.132 - 0.303	0.12 - 0.51	0.0948 - 0.272
	Range	0.124 - 0.228	0.126 - 0.239			
	Confidence Interval	0.177 - 0.204	0.188 - 0.214			
	Adjusted P-Value	--	0.468			
	P-Value	--	0.206			
Glutamic Acid	Mean	1.53	1.57	1.04 - 2.70	0.97 - 3.54	1.24 - 2.38
	Range	1.23 - 2.03	1.20 - 2.03			
	Confidence Interval	1.38 - 1.68	1.42 - 1.71			
	Adjusted P-Value	--	0.459			

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
	P-Value	--	0.127			
Glycine	Mean	0.350	0.362	0.292 - 0.487	0.184 - 0.685	0.291 - 0.446
	Range	0.304 - 0.392	0.303 - 0.461			
	Confidence Interval	0.332 - 0.367	0.344 - 0.379			
	Adjusted P-Value	--	0.215			
	P-Value	--	0.00731[a]			
Histidine	Mean	0.249	0.256	0.177 - 0.359	0.14 - 0.46	0.200 - 0.345
	Range	0.206 - 0.300	0.207 - 0.297			
	Confidence Interval	0.231 - 0.267	0.238 - 0.274			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.0819			
Isoleucine	Mean	0.282	0.289	0.229 - 0.494	0.18 - 0.69	0.237 - 0.421
	Range	0.231 - 0.389	0.223 - 0.386			
	Confidence Interval	0.256 - 0.308	0.263 - 0.315			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.173			
Leucine	Mean	1.01	1.03	0.763 - 1.85	0.64 - 2.49	0.843 - 1.62
	Range	0.802 - 1.46	0.778 - 1.45			
	Confidence Interval	0.898 - 1.12	0.920 - 1.15			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.168			
Lysine	Mean	0.263	0.272	0.186 - 0.412	0.129 - 0.668	0.127 - 0.391
	Range	0.198 - 0.319	0.220 - 0.327			
	Confidence Interval	0.246 - 0.279	0.256 - 0.288			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.146			
Methionine	Mean	0.187	0.201	0.108 - 0.342	0.10 - 0.47	0.104 - 0.246
	Range	0.135 - 0.231	0.143 - 0.234			
	Confidence Interval	0.174 - 0.200	0.188 - 0.214			
	Adjusted P-Value	--	0.334			
	P-Value	--	0.0246[a]			
Phenylalanine	Mean	0.418	0.430	0.342 - 0.736	0.24 - 0.93	0.321 - 0.626
	Range	0.293 - 0.570	0.314 - 0.567			
	Confidence Interval	0.371 - 0.465	0.383 - 0.477			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.189			

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

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

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

Minerals in DP202216 Maize Grain

Minerals were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in **Tables 16** and **17**. 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-GM near-isoline control maize and non-GM commercial maize.

Table 16. Mineral Results for DP202216 Maize in Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
Calcium	Mean	0.00342	0.00340	0.00131 - 0.00784	ND - 0.101	0.00212 - 0.00595
	Range	0.00285 - 0.00435	0.00271 - 0.00408			
	Confidence Interval	0.00321 - 0.00364	0.00318 - 0.00361			
	Adjusted P-Value	--	0.875			
	P-Value	--	0.720			
Copper	Mean	0.000128	0.000125	<0.0000625 - 0.000617	ND - 0.0021	<0.0000625[a] - 0.000169
	Range	<0.0000625[a] - 0.000238	<0.0000625[a] - 0.000212			
	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	0.00118 - 0.00261	0.0000712 - 0.0191	0.00120 - 0.00218
	Range	0.00151 - 0.00195	0.00146 - 0.00220			
	Confidence Interval	0.00160 - 0.00177	0.00164 - 0.00181			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.168			
Magnesium	Mean	0.108	0.110	0.0787 - 0.163	0.0035 - 1.000	0.0820 - 0.147
	Range	0.0876 - 0.137	0.0904 - 0.136			
	Confidence Interval	0.0993 - 0.116	0.102 - 0.119			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.188			
Manganese	Mean	0.000556	0.000571	0.000328 - 0.00131	0.0000312 - 0.0054	0.000289 - 0.000992
	Range	0.000346 - 0.000801	0.000273 - 0.000850			
	Confidence Interval	0.000426 - 0.000685	0.000442 - 0.000701			
	Adjusted P-Value	--	0.468			
	P-Value	--	0.204			
Phosphorus	Mean	0.296	0.298	0.204 - 0.429	0.010 - 0.750	0.189 - 0.410
	Range	0.209 - 0.367	0.205 - 0.351			
	Confidence Interval	0.262 - 0.330	0.264 - 0.332			
	Adjusted P-Value	--	0.878			

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
	P-Value	--	0.775			
Potassium	Mean	0.399	0.395	0.222 - 0.541	0.18 - 0.720	0.276 - 0.511
	Range	0.306 - 0.459	0.316 - 0.451			
	Confidence Interval	0.371 - 0.427	0.367 - 0.423			
	Adjusted P-Value	--	0.836			
	P-Value	--	0.615			
Sodium	Mean	0.000158	0.000101	0.00000298 - 0.00366	ND - 0.150	<0.0000625 [a] - 0.00207
	Range	<0.0000625[a] - 0.000961	<0.0000625[a] - 0.000726			
	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	0.00140 - 0.00365	0.0000283 - 0.0043	0.00150 - 0.00295
	Range	0.00183 - 0.00277	0.00166 - 0.00282			
	Confidence Interval	0.00205 - 0.00248	0.00205 - 0.00248			
	Adjusted P-Value	--	0.993			
	P-Value	--	0.993			

Note: Minerals unit of measure is % dry weight. Not detectable (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 17. Number of Minerals Sample Values Below the Lower Limit of Quantification for DP202216 Maize in 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.

Vitamins in DP202216 Maize Grain

Vitamins were analyzed in grain derived from DP202216 maize and near-isoline control maize. Results are shown in **Table 18** and **19**. 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 of 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-GM near-isoline control maize and non-GM commercial maize.

Table 18. Vitamin Results for DP202216 Maize in Grain

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
β-Carotene	Mean	0.983	0.962	<0.0500 - 2.06[a]	0.3 - 5.4	0.249 - 3.51
	Range	0.429 - 2.08	0.413 - 2.30			
	Confidence Interval	0.615 - 1.35	0.593 - 1.33			
	Adjusted P-Value	--	0.815			
	P-Value	--	0.503			
Vitamin B1 (Thiamine)	Mean	2.38	2.54	1.71 - 5.38	ND - 40.00	1.97 - 3.11
	Range	2.08 - 3.08	1.99 - 3.23			
	Confidence Interval	2.25 - 2.51	2.41 - 2.68			
	Adjusted P-Value	--	0.215			
	P-Value	--	0.00466[b]			
Vitamin B2 (Riboflavin)	Mean	<0.900[c]	<0.900[c]	<0.900 - 2.27[a]	ND - 7.35	<0.900[c]
	Range	<0.900[c]	<0.900[c]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Vitamin B3 (Niacin)	Mean	14.7	13.5	7.86 - 25.2	ND - 70	9.49 - 66.0
	Range	10.9 - 22.7	9.33 - 16.2			
	Confidence Interval	13.9 - 15.6	12.7 - 14.4			
	Adjusted P-Value	--	0.215			
	P-Value	--	0.00947[b]			
Vitamin B5 (Pantothenic Acid)	Mean	5.11	4.71	3.05 - 7.66	3.0 - 14	3.08 - 6.51
	Range	3.62 - 7.10	3.16 - 6.22			
	Confidence Interval	4.66 - 5.57	4.25 - 5.17			
	Adjusted P-Value	--	0.459			
	P-Value	--	0.152			
Vitamin B6 (Pyridoxine)	Mean	4.54	4.44	1.37 - 8.67	ND - 12.14	2.51 - 10.7
	Range	2.81 - 9.48	2.23 - 8.15			
	Confidence Interval	3.95 - 5.22	3.87 - 5.11			
	Adjusted P-Value	--	0.878			
	P-Value	--	0.761			
Vitamin B9 (Folic Acid)	Mean	0.923	0.854	0.319 - 2.41	ND - 3.50	0.461 - 2.70
	Range	0.565 - 2.50	0.235 - 1.72			
	Confidence Interval	0.795 - 1.07	0.735 - 0.992			
	Adjusted P-Value	--	0.794			
	P-Value	--	0.456			

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range
α-Tocopherol	Mean	4.28	4.44	0 - 25.1	ND - 68.67	<0.500[c] - 21.3
	Range	0.969 - 7.63	1.07 - 8.92			
	Confidence Interval	3.08 - 5.48	3.24 - 5.64			
	Adjusted P-Value	--	0.830			
	P-Value	--	0.574			
β-Tocopherol	Mean	<0.500[c]	<0.500[c]	<0.500 - 1.10[a]	ND - 19.80	<0.500[c]
	Range	<0.500[c]	<0.500[c]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
γ-Tocopherol	Mean	25.9	26.9	0 - 46.5	ND - 58.61	3.06 - 42.7
	Range	10.8 - 35.6	11.4 - 36.3			
	Confidence Interval	21.9 - 30.0	22.8 - 30.9			
	Adjusted P-Value	--	0.740			
	P-Value	--	0.392			
δ-Tocopherol	Mean	0.519	0.533	<0.500 - 2.61[a]	ND - 14.61	<0.500[c] - 1.14
	Range	<0.500[c] - 1.16	<0.500[c] - 1.13			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Total Tocopherols	Mean	31.0	32.1	0 - 61.0	ND - 89.91	5.33 - 52.1
	Range	12.3 - 42.2	13.6 - 42.8			
	Confidence Interval	26.7 - 35.3	27.8 - 36.4			
	Adjusted P-Value	--	0.788			
	P-Value	--	0.438			

Note: Vitamins unit of measure is mg/kg dry weight. Not detectable (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] 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 19. Number of Vitamins Sample Values Below the Lower Limit of Quantification for DP202216 Maize in 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.

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 20** and **21**. 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-GM near-isoline control maize and non-GM commercial maize.

Table 20. Secondary Metabolites and Anti-Nutrients Results for DP202216 Maize in Grain

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

Analyte	Reported Statistics	Control Maize	DP202216 Maize	Tolerance Interval	Literature Range	Reference Data Range

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 21. Number of Secondary Metabolites and Anti-Nutrients Sample Values Below the Lower Limit of Quantification for DP202216 Maize in 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 the control maize and was subjected to the mixed model analyses.

Nutrient Composition Assessment Conclusion

In conclusion, the results of the compositional analysis demonstrated that nutrient composition of forage and grain derived from DP202216 maize was comparable to that of conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

C Information related to the nutritional impact of the food

As seen in above Section B5, the compositional analysis did not indicate any biologically significant changes to the levels of nutrients in the food produced gene technology compared to the non-GM counterpart food. Therefore, a dietary exposure analysis (DEA) has not been submitted.

DP202216 expresses *ZMM28*, a maize-endogenous protein. The expression levels in grain were at the ppb level (mean: 0.012 ng/mg; range: <0.0069-0.029) with a quarter of the samples being below the LOQ.

DP202216 also expresses the PAT protein. PAT has had its safety assessed(Hérouet et al. 2005) in more than 20 previous FSANZ applications, is globally represented in six major crop species and in more than 30 approved GM single plant events (FAO GM Foods Platform).

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Appendix A. Refer to Attachment 2: Commercial in Confidence Information

Appendix B. Methods for Southern-by-Sequencing Analysis ([redacted] 2019)

A. Sample Collection

Seeds of DP202216 maize and control maize were planted, grown, and leaf tissue was collected. The leaf samples used for DNA extraction and SbS analysis were maintained frozen (≤ -50 °C) until processing.

B. Polymerase Chain Reaction Analysis of Plants

After germination and prior to tissue sampling for DNA extraction, all plants were analysed by polymerase chain reaction (PCR). 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).

C. 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 6**, Step 1).

D. Southern-by-Sequencing

SbS was performed by Corteva 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 T1 generation of DP202216 maize were analysed 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.

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

1. **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 6**, Step 2).

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 analysed using a fragment analyser and diluted to 5 ng/µl with nuclease-free water (**Figure 6**, Step 3).

3. **Probe Hybridization and Sequence Enrichment**

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

4. **Next Generation Sequencing on Illumina Platform**

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

5. **Quality Assurance of Sequencing Reads**

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

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 6**, Step 7). Due to the detection of these bacterial sequences, coverage levels of 35x or below were considered to be the background level of sequencing.

7. 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 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 6**, Step 8).

8. Junction Identification

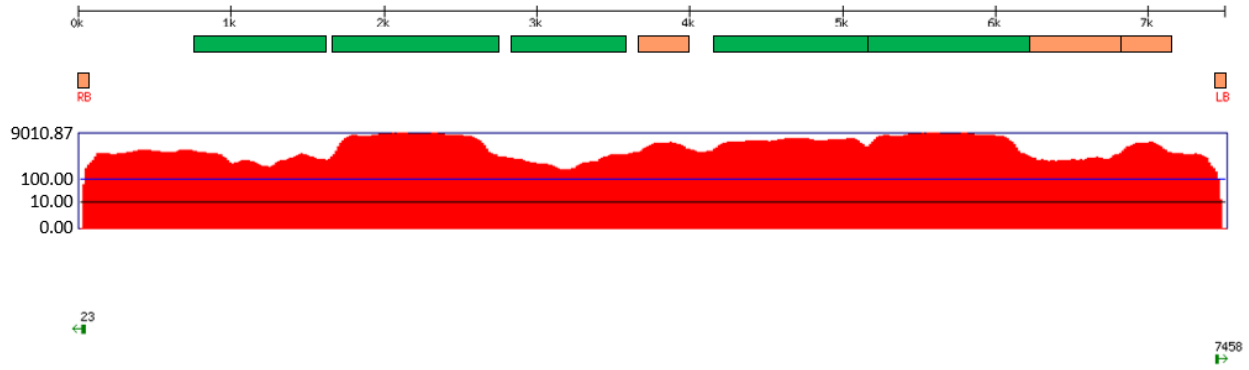
Variations between the maize reference genome and the sequences of endogenous maize sequences that are found in the transformation construct may result in identification of junctions that are due to these endogenous maize sequences. In order to detect these endogenous junctions, control maize genomic DNA libraries were captured and sequenced in the same manner. These libraries were sequenced to an average depth approximately five times 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 6**, Step 8), so that the only junctions remaining in the DP202216 samples are due to actual PHP40099 insertions (**Figure** 6, Step 9).

E. SbS Results

Results for the control maize, positive control, and "representative plant" (Plant ID 335728647) are presented in the main body (Section A.3) of this document.

Remaining plant results from SbS analysis follow:

A.



B.

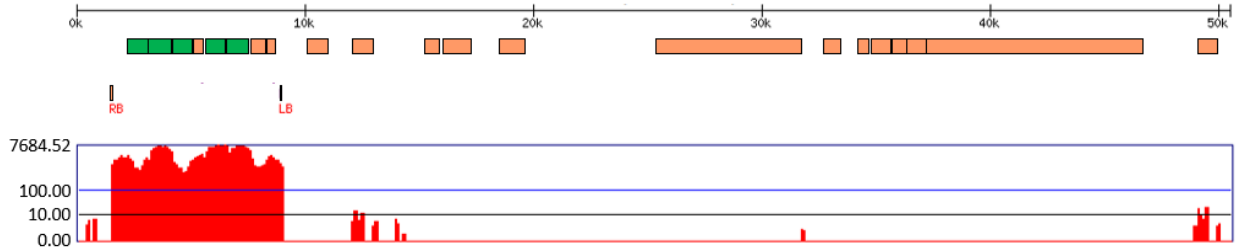
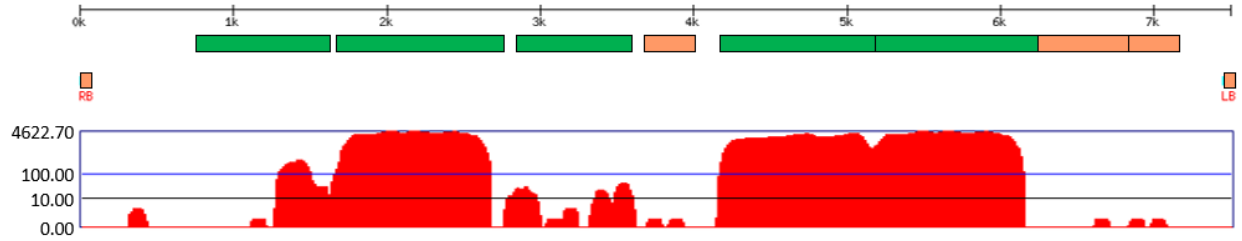


Figure B1. SbS Results for DP202216 Maize (Plant ID 335728648)

This sample was positive for the DP202216 insertion as confirmed by PCR (**Table 3**). The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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. Green arrows indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the 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 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 backbone sequence present in DP202216 maize.

A.



B.

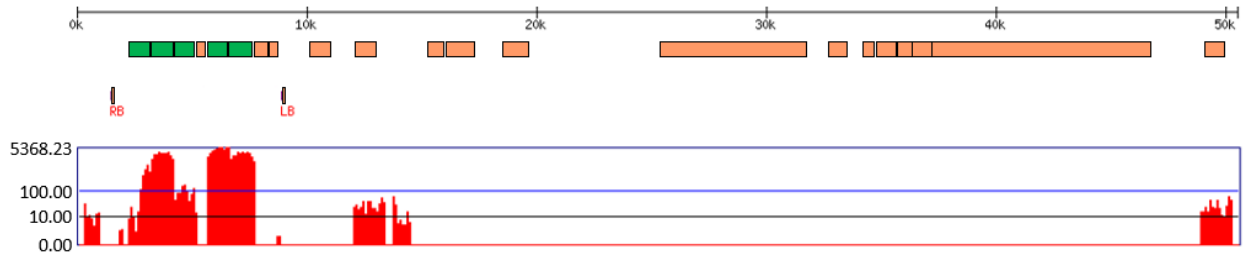
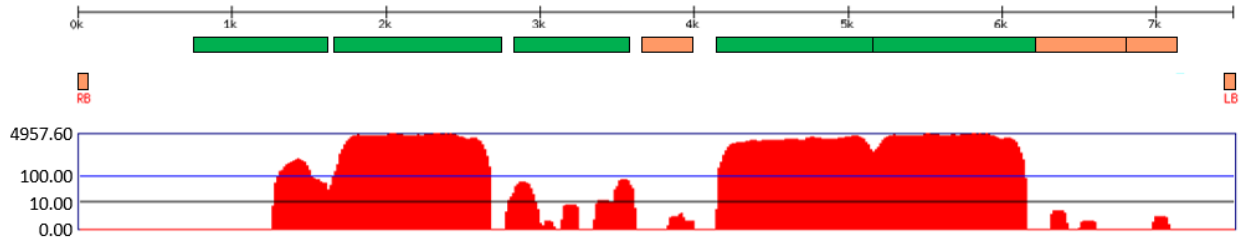


Figure B2. SbS Results for DP202216 Maize (Plant ID 335728649)

This sample was negative for the DP202216 insertion as confirmed by PCR (**Table 3**). The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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 PH17AW control maize (**Figure 9**) 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 indicates that there are no insertions or backbone sequence present in this plant.

A.



B.

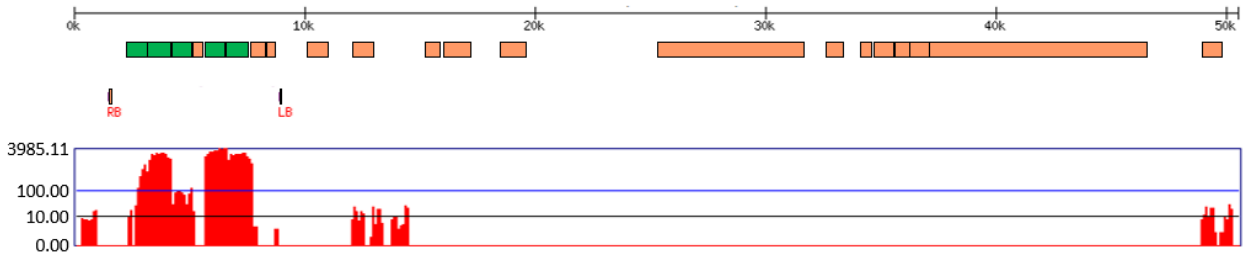
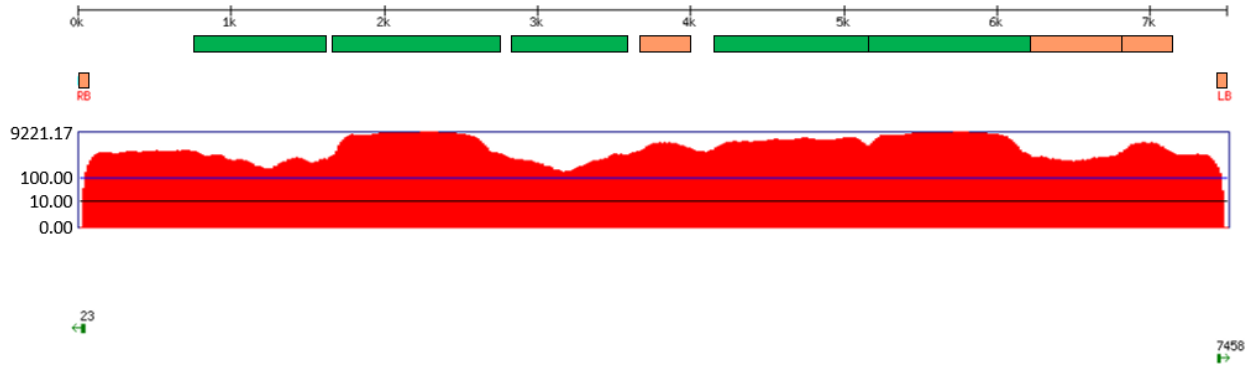


Figure B3. SbS Results for DP202216 Maize (Plant ID 335728650)

This sample was negative for the DP202216 insertion as confirmed by PCR (**Table 3**). The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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 PH17AW control maize (**Figure 9**) 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 indicates that there are no insertions or backbone sequence present in this plant.

A.



B.

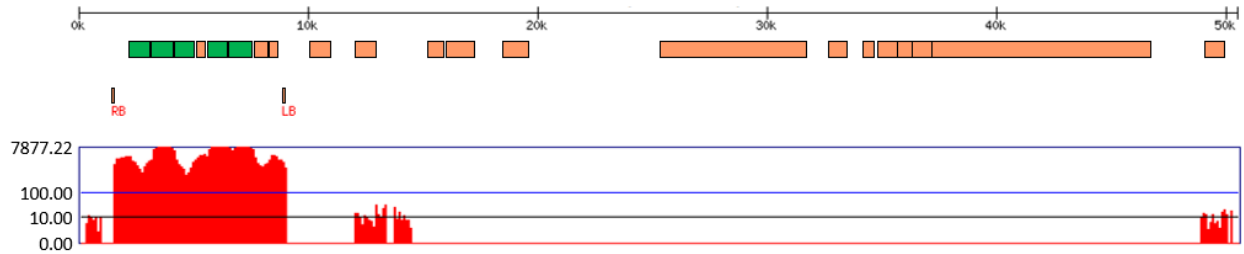
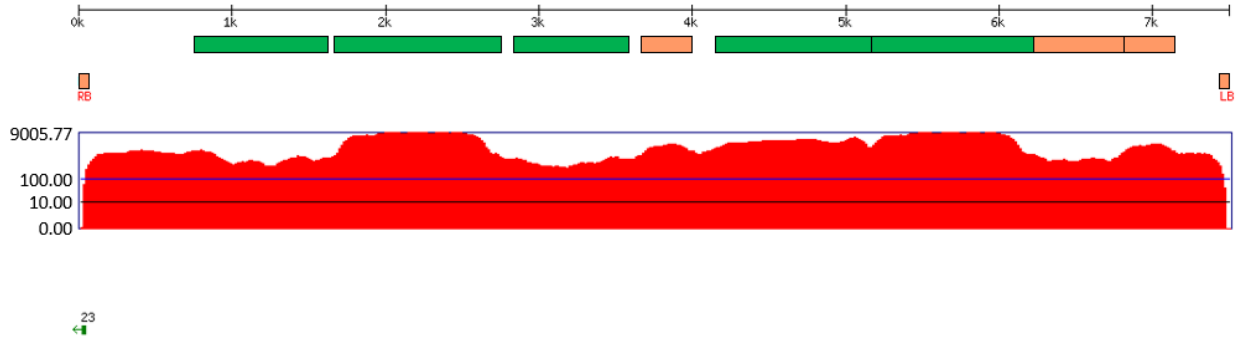


Figure B4. SbS Results for DP202216 Maize (Plant ID 335728651)

This sample was positive for the DP202216 insertion as confirmed by PCR (**Table 3**). The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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. Green arrows indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the 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 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 backbone sequence present in DP202216 maize.

A.



B.

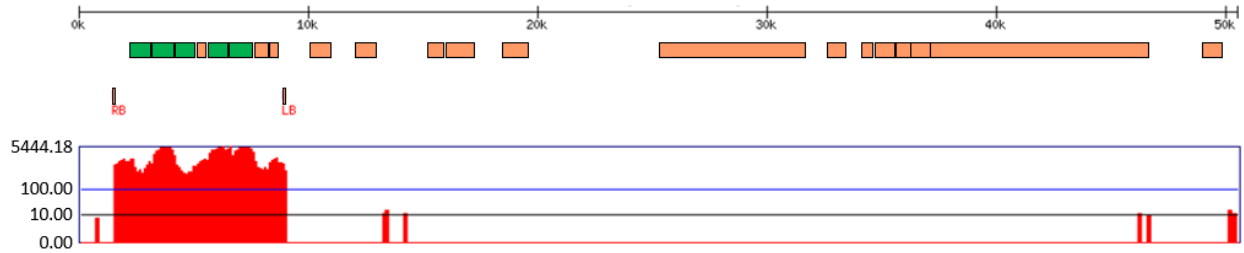
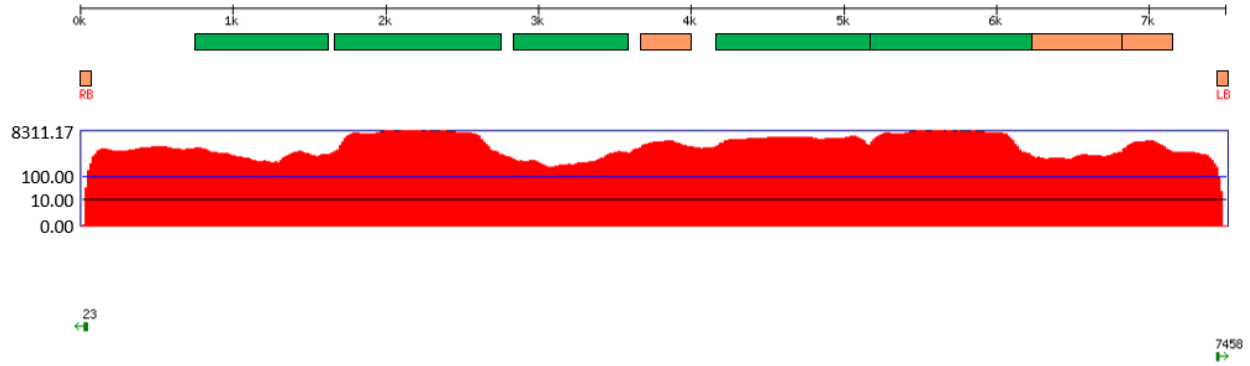


Figure B5. SbS Results for DP202216 Maize (Plant ID 335728652)

This sample was positive for the DP202216 insertion as confirmed by PCR (**Table 3**). The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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. Green arrows indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the 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 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 backbone sequence present in DP202216 maize.

A.



B.

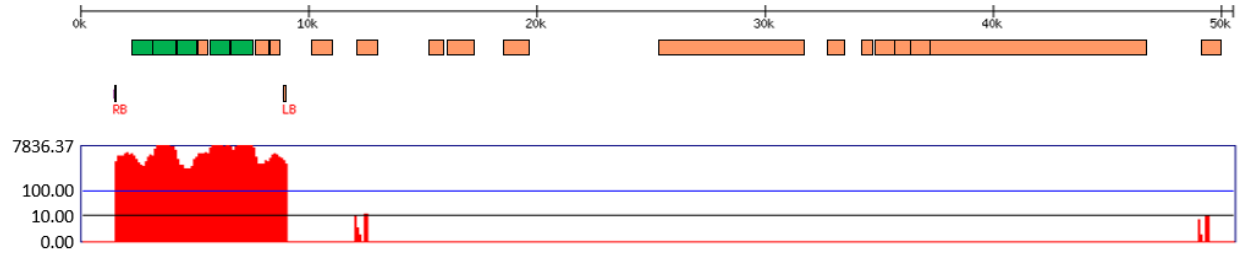
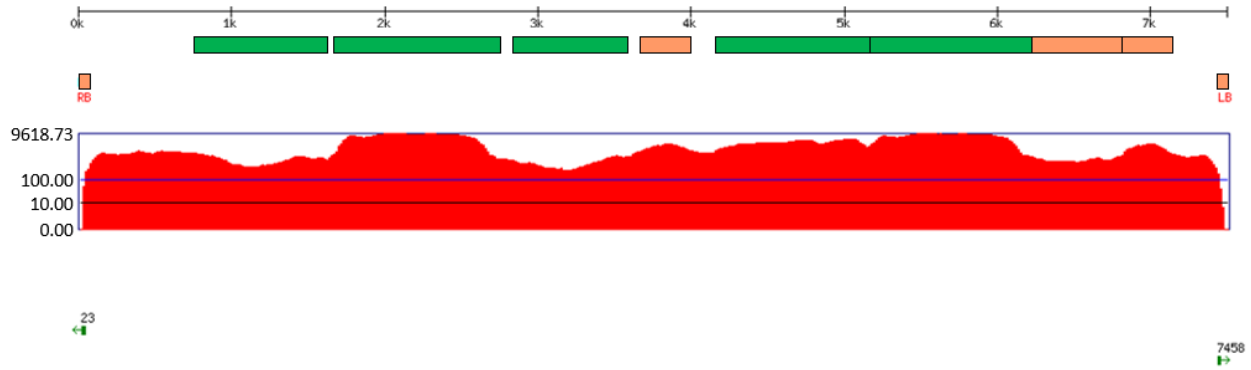


Figure B6. SbS Results for DP202216 Maize (Plant ID 335728653)

This sample was positive for the DP202216 insertion as confirmed by PCR (**Table 3**). The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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. Green arrows indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the 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 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 backbone sequence present in DP202216 maize.

A.



B.

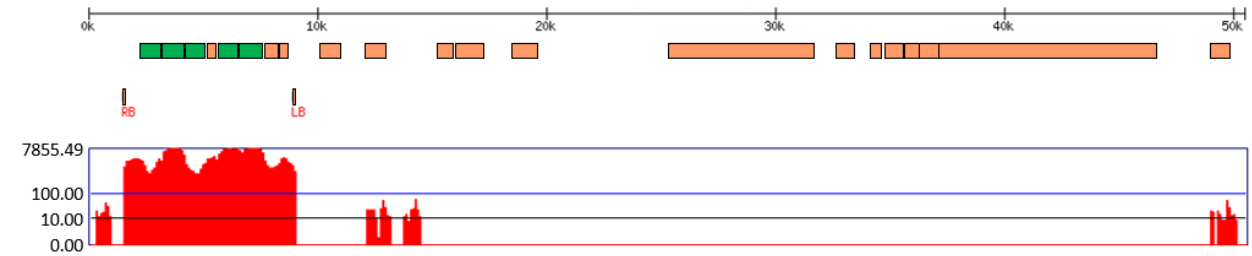


Figure B7. SbS Results for DP202216 Maize (Plant ID 335728654)

This sample was positive for the DP202216 insertion as confirmed by PCR (**Table 3**). The red coverage graph shows the number of individual sequence reads aligned at each point on the construct using a logarithmic scale. Green bars above the coverage graph indicate 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. Green arrows indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers above the arrows refer to the bp location of the junction relative to the 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 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 backbone sequence present in DP202216 maize.

Appendix C. Methods for Southern Analysis [redacted] 2018)

A. Sample Collection

Genomic DNA was isolated and analysed from leaf tissue from five generations (one plant from each of the T1, T2, BC1F1, BC3F3, and BC3F6 generations) of DP202216 maize and one plant from each of the PH17AW and PHR1J control maize lines.

B. DNA Extraction and Quantitation

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.

C. 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).

D. 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).

E. Probe Labelling and Southern Blot Hybridization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labelled probe. DNA probes specific to the *zmm28* and *mo-pat* genes (**Figure 12**) were labelled by incorporation of Digoxigenin (DIG) labelled nucleotide [DIG-11-dUTP] into the fragments. Detailed descriptions of these probes are provided in **Table 4**.

Labelled 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) labelled (Roche, Indianapolis, IN, USA) were used for visualization as the fragment size standards on the blot.

F. Detection of Hybridized Probes

After stringent washes, DIG-labelled DNA standards and single stranded DIG-labelled probes hybridized to DNA bound to the nylon membrane were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche, 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.

G. Stripping of Probes and Subsequent Hybridizations

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

Appendix D. Methods for Multi-Generation Segregation Analysis [redacted] 2018)

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

A. Greenhouse Experimental Design

Five separate generations (T2, F1(PH17AW/PHR1J), BC1F1, BC3F3, and BC3F6; **Figure 3**) 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 analysed using PCR amplification methods specific for the event DP-2Ø2216-6, *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 tolerance.

B. 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 labelled to allow a given sample to be tracked back to the originating plant.

C. Genotypic Analysis

Leaf samples were analysed using an event-specific PCR assay to confirm the presence or absence of event DP-2Ø2216-6, and gene-specific PCR assays to confirm the presence or absence of the *mo-pat* and *zmm28* genes.

D. Phenotypic Analysis

For the F1(PH17AW/PHR1J), BC1F1, BC3F3, T2, and BC3F6 generations, glufosinate 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 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 tolerance in which presence of herbicide injury corresponded to an herbicide-susceptible phenotype and absence of herbicide injury corresponded to an herbicide-tolerant phenotype.

E. Statistical Analysis

A chi-square analysis was performed T-DNA Region from 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 T2 and 1:1 for F1(PH17AW/PHR1J) and BC1F1). 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 BC3F3 and BC3F6 because all plants were identified as positive (*i.e.*, not segregating) as expected for a homozygous generation.

Appendix E. Methods for Protein Characterization and Equivalency Analyses

A. *ZMM28* Protein Western Blot Method ([redacted] 2018)

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 control maize for western blot analysis. Samples were lyophilized, processed, and stored at ≤ -10 °C.

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 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. 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.

B. PAT Protein Western Blot Method ([redacted] 2018)

Protein Sample Preparation and Extraction

Leaf (V9 growth stage; Abendroth *et al.*, 2011) samples were collected from the DP202216 maize and near-isoline control maize lines for western blot analysis. Samples were lyophilized, processed, and stored at ≤ -10 °C.

The lyophilized leaf samples were weighed into 1.2-ml tubes at a target weight of 10 mg (± 5%). Tissue 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. Prestained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into the gel to provide a visual verification that migration was within the range of the predicted molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES SDS running buffer 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 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 F. 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 analysed by SDS-PAGE.

Appendix G. 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 [redacted] 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 H. Methods for Expressed Trait Protein Analysis [redacted] 2018)

A. Field Trial Experimental Design

The field portion of this study was conducted during the 2017 growing season at six sites in commercial 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.

B. 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 labelled with a sample identification number and barcode for sample tracking, and is traceable by site, entry, block, tissue, and growth stage.

1. 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-labelled, 50-ml vial.

2. 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-labelled, 50-ml vial.

3. 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 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-labelled, 50-ml vial.

4. 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-labelled, plastic-lined, cloth bag. The plants selected for forage sampling contained self-pollinated ears.

5. 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-labelled, 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.

6. 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-labelled, 35-ml vial.

C. Sample Processing, Shipping, and Storage

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 Corteva Agriscience 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.

D. 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.

1. 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, root, forage, and whole plant sample analysed 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 analysed for *ZMM28* protein concentration was extracted in 0.60 ml of lithium dodecyl sulfate with dithiothreitol (LDS/DTT) extraction buffer. Samples analysed 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.

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

Prior to analysis, leaf, pollen, root, forage, and whole plant samples were diluted as applicable in PBST with 0.25% ASB-14. Standards (typically analysed in triplicate wells) and diluted samples (typically analysed 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 coloured 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.

3. *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 analysed in single lanes), grain samples (typically analysed 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. 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 analysing 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).

4. PAT Protein ELISA Method for Maize Tissues

Prior to analysis, samples were diluted as applicable in PBST. Standards (typically analysed in triplicate wells) and diluted samples (typically analysed 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 coloured 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.

A. 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.

Adjusted Concentration = Interpolated Sample Concentration x Dilution Factor

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

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

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

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

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

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

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

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

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

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

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

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

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

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

B. 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.

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

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:

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

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:

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

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

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

C. Statistical Analysis

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

Appendix I. Methods for Nutrient Composition Analysis ([redacted] 2018)

A. Field Trial Experimental Design

The field portion of this study was conducted during the 2017 growing season at eight sites in commercial maize-growing regions of the United States (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 reference maize. A randomized complete block design with four blocks was utilized at each site.

B. Sample Collection

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

1. 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-labelled, plastic-lined, cloth bag.

2. 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-labelled, plastic, resealable bag and then placed into a pre-labelled, plastic-lined, cloth bag.

Each forage and grain sample were 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 Corteva Agriscience and then shipped frozen to EPL Bio Analytical Services (EPL BAS, Niantic, IL, USA) for analyses, or shipped frozen directly to EPL BAS.

D. 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 C.1**. Nutrient composition analyses of forage and grain samples included the determination of the following analytes:

1. Proximates, Fibre, and Minerals Composition in Forage

- Moisture*
- Crude Protein
- Crude Fat
- Crude Fibre
- Acid Detergent Fibre (ADF)
- Neutral Detergent Fibre (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.

2. Proximates and Fibre Composition in Grain

- Moisture*
- Total Dietary Fibre
- Crude Protein
- Crude Fat
- Crude Fibre
- Acid Detergent Fibre (ADF)
- Neutral Detergent Fibre (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.

3. 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)

4. 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

5. Mineral Composition in Grain

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

6. Vitamin Composition in Grain

- β-Carotene
- Vitamin B1 (Thiamine)
- Vitamin B2 (Riboflavin)
- Vitamin B9 (Folic Acid)
- α-Tocopherol
- β-Tocopherol

- Vitamin B3 (Niacin)
- Vitamin B5 (Pantothenic Acid)
- Vitamin B6 (Pyridoxine)
- 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.

7. Secondary Metabolite and Anti-Nutrient Composition in Grain

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

Table I.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 analysed 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 Kjeltec Analyzer unit.
Crude Fat Forage and Grain	The analytical procedure for crude fat determination was based on methods provided by the American Oil Chemists' Society (AOCS) and the manufacturer of the hydrolysis and extraction apparatus (Ankom Technology). Samples were hydrolysed with 3N hydrochloric acid at 90 °C for 80 minutes for forage and 60 minutes for grain. The hydrolysates were extracted with a petroleum ether/ethyl ether/ethyl alcohol solution at 90 °C for 60 minutes. The ether extracts were evaporated and the fat residue remaining determined gravimetrically.
Carbohydrates Forage and Grain	The carbohydrate content in maize forage and grain on a dry weight basis was calculated using a formula obtained from the United States Department of Agriculture "*Energy Value of Foods,*" in which the percent dry weight of crude protein, crude fat, and ash was subtracted from 100%.
Crude Fibre Forage and Grain	The analytical procedure for crude fibre determination was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC, and the AOCS. Samples were analysed to determine the percentage of crude fibre by digestion and solubilization of other materials present.
Neutral Detergent Fibre	The analytical procedure for neutral detergent fibre (NDF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology), the AOAC, and the *Journal of AOAC International*. Samples were analysed 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 Fibre Forage and Grain	The analytical procedure for acid detergent fibre (ADF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology) and the AOAC. Samples were analysed 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 I.1. Methods for Compositional Analysis of DP202216 Maize (continued)

Nutritional Analyte	Method
Total Dietary Fibre	The analytical procedure for the determination of total dietary fibre 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 fibre 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 analysed 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 analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES).
Tryptophan	The analytical procedure for tryptophan determination was based on an established lithium hydroxide hydrolysis procedure with reverse phase ultra-performance liquid chromatography (UPLC) with ultraviolet (UV) detection published by the *Journal of Micronutrient Analysis*.
Cystine and Methionine	The analytical procedure for cystine and methionine determination was based on methods obtained from Waters Corporation, AOAC, and *Journal of Chromatography A*. The procedure converts cystine to cysteic acid and methionine to methionine sulfone, after acid oxidation and hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives which are then analysed by reverse phase UPLC with UV detection.
Additional Amino Acids	Along with tryptophan, cystine, and methionine, 15 additional amino acids were determined. The analytical procedure for analysis of these amino acids was based on methods obtained from Waters Corporation and the *Journal of Chromatography A*. The procedure converts the free acids, after acid hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives, which are analysed 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 analysed 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 analysed by reverse phase high pressure liquid chromatography (HPLC) tandem mass spectrometry (MS/MS).

Table I.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 *Lactobacillus plantarum* and allowed to incubate for approximately 18 to 22 hours. After incubation, the bacterial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of nicotinic acid.
Pantothenic Acid (Vitamin B5)	The analytical procedure for the determination of pantothenic acid (vitamin B5) was based on a method from the AOAC. Pantothenic acid (vitamin B5) was determined using a microbiological assay. Pantothenic acid (vitamin B5) was extracted from the sample by adding an acetic acid buffer solution and autoclaving. The pH was adjusted, and a tube array was prepared using three different dilutions of the samples. This tube array was inoculated with *Lactobacillus plantarum* and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of D-pantothenic acid hemicalcium salt.
Pyridoxine (Vitamin B6)	The analytical procedure for the determination of pyridoxine (vitamin B6) was based on a method from the AACC. Pyridoxine (vitamin B6) was determined using a microbiological assay. Pyridoxine (vitamin B6) was extracted from the sample by adding sulfuric acid and autoclaving. The pH was adjusted, and a tube array was prepared using four different dilutions of the samples. This tube array was inoculated with *Saccharomyces cerevisiae* and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 600 nm. The absorbance readings were compared to a standard curve generated using known concentrations of pyridoxine hydrochloride.
Total Folate as Folic Acid (Vitamin B9)	The analytical procedure for determination of total folate as folic acid was based on a microbiological assay published by the AACC. Samples were hydrolysed 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 *Lactobacillus casei*. The total folate content was determined by measuring the turbidity of the *Lactobacillus casei* growth response in the sample and comparing it to the turbidity of the growth response with folic acid standards using a spectrophotometer at 600 nm.

Table I.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 *Journal of the American Oil Chemists' Society* and *Analytical Sciences*. Alpha, beta, gamma, and delta tocopherols were extracted with hot hexane and the extracts were analysed 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 analysed 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 *Handbook of Analytical Derivatization Reactions*, an AACC method, and a method from the *Journal of Agricultural and Food Chemistry*. 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 *Journal of Agricultural and Food Chemistry*. Ground maize grain was analysed for furfural content by reverse phase HPLC with UV detection.
p-Coumaric and Ferulic Acid	The analytical procedure for the determination of *p*-coumaric and ferulic acids was developed based on methods published in *Journal of Agricultural and Food Chemistry* and *The Journal of Chemical Ecology*. Ground maize grain was analysed to determine the amounts of *p*-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 analysed 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 analysed for elemental phosphorus by ICP-OES.

E. 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.

1. Processing of Data

a. 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.

b. Conversion of Fatty Acid Assay Values

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

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

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

c. 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.

2. 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 ≥ 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.

3. Statistical Model for Across-Site Analysis

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

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

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

4. 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 analysed 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.

5. **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 Corteva's proprietary accumulated data from non-GM maize lines, which were grown in commercial maize-growing regions in the United States, Canada, and South America between 2003 and 2015. The combined data represent 93 commercial maize lines and 88 unique

environments. The selected commercial maize lines represent the non-GM maize population with a history of safe use, and the selected environments (site and year combinations) represent maize growth under a wide range of environmental conditions (*i.e*. soil texture, temperature, precipitation, and irrigation) and maize maturity group zones.

If the range of 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-GM commercial maize lines and growing environments, which are not included in Corteva'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 non-GM reference maize lines grown in this study. In-study reference data ranges compliment tolerance intervals and literature ranges in that they provide additional context of natural variation specific to the current study.

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

6. **Reported Statistics**

The outcome of the nutrient composition assessment is provided in **Table 10**. 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) (labelled as Mean, Range, and Confidence Interval, respectively) are provided in **Tables 11-21** for the across-site analysis. Both the FDR-adjusted P-values and non-adjusted P-values (labelled 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 analysed 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.