Application to Amend the Australia New Zealand Food Standards Code

Schedule 26 - ***Food Produced Using Gene Technology***

OECD Unique Identifier - DP-Ø51291-2

DP51291 Maize

Submitting company:

Corteva Agriscience Australia Pty Ltd

Submitted by:

January 2023

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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.

Corteva Agriscience Australia Pty Ltd, member of Corteva Agriscience group of companies, is submitting this application to FSANZ to vary the Code to approve uses of insect-resistant and herbicide-tolerant maize (*Zea mays L.*) event DP- Ø51291-2 (referred to as DP51291 maize), a new food produced using gene technology.

DP51291 maize was genetically modified to express the IPD072Aa protein for control of susceptible corn rootworm (CRW) pests, the phosphinothricin acetyltransferase (PAT) protein for tolerance to glufosinate herbicide, and the phosphomannose isomerase (PMI) protein that was used as a selectable marker. The IPD072Aa protein was previously evaluated in corn line DP23211 (Application A1202). The PAT and PMI proteins are found in several approved events that are currently in commercial use.

This application presents information supporting the safety and nutritional comparability of DP51291 maize. The molecular characterization analyses conducted on DP51291 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 allergenic and toxic potential of the IDP072Aa, PAT and PMI proteins was evaluated previously, and these proteins were found unlikely to be allergenic or toxic to humans or animals. In accordance with the Application Handbook, only the updated bioinformatics analyses are provided for these proteins for safety assessment within this application. A compositional equivalence assessment demonstrated that the nutrient composition of DP51291 maize forage and grain is comparable to that of conventional maize, represented by non-genetically modified (non-GM) near-isoline maize and non-GM commercial maize.

Overall, data and information contained herein support the conclusion that DP51291maize containing the IPD072Aa, PAT, and PMI proteins is as safe and nutritious as non-GM maize for food and feed uses.

TABLE OF CONTENTS

Summary 2
Table of Contents 3
List of Tables 5
List of Figures 6
Checklists 8
Statutory Declaration 10
General information on the application 11
B. Applicant 11
C. Purpose of the application 11
D. Justification for the application 11
D(a) Need for the proposed change 11
D(b) Advantage of the genetically modified food 12
D.1 Regulatory impact 12
A Technical information on the food produced using gene technology 14
A.1 Nature and identity of the genetically modified food 14
A.1(a) Description of the GM organisms, nature and purpose of the genetic modification 14
A.1(b) GM organism identification 14
A.1(c) Trade name 14
A.2 History of use of the host and donor organisms 15
A.2(a) Donor organisms 15
A.2(b) Host organism 16
A.3 The nature of the genetic modification 17
A.3(a) Transformation method 17
A.3(b) Construct and the transformation vectors used 41
A.3(c) Molecular characterisation 41
A.3(d) Selection and breeding process 66
A.3(e) Stability of genetic changes 68
B Characterisation and safety assessment of the new substances 70
B.1 Characterisation and safety assessment of new substances 70
B.2 New proteins 70
IPD072Aa protein 70
PAT protein 79
PMI protein 87
B.3 Other (non-protein) new substances 95
B.4 Novel herbicide metabolites in GM herbicide-tolerant plants 95
B.5 Compositional analyses 95
C Information related to the nutritional impact 121
D Other information 122
Overall risk assessment conclusions for DP51291 maize 122
References 123
Study index 132
Appendix A. Methods for Southern-by-Sequencing Analysis (2022 (PHI-2022-120 study)) 134
Appendix B. Methods for Southern Blot Analysis (, 2022 (PHI-2022-064 study)) 149
Appendix C. Methods for Multi-Generation Segregation Analysis (et al., 2022 (PHI-2018-035 study)) 151
Appendix D. Methods for Characterisation of IPD072Aa Protein (et al., 2022 (PHI-2022-054 study))

.... 153
Appendix E. Methods for Characterisation of PAT Protein (et al., 2022 (PHI-2022-055 study)) 156
Appendix F. Methods for Characterisation of PMI Protein (et al., 2022 (PHI-2022-123 study)) 159
Appendix G. Methods for Expressed Trait Analyses et al., 2022 (PHI-2021-034 study)) 162
Appendix H. Methods for Nutrient Composition Analysis et al., 2019b (PHI-2021-035/021 study)) . 166

LIST OF TABLES

Table 1. List of Relevant Genetic Elements in the T-DNA Region of Plasmid [redacted] Used in the First Transformation and Their Presence in DP51291 Maize 19
Table 2. List of Relevant Genetic Elements in Plasmids Used in the Second Transformation and Their Presence in DP51291 Maize 19
Table 3. List of Relevant Genetic Elements in the T-DNA Region of Plasmid [redacted] Used in the Third Transformation and Their Presence in DP51291 Maize 19
Table 4. Description of the Genetic Elements in Plasmid [redacted] 35
Table 5. Description of the Genetic Elements in the T-DNA Region from Plasmid [redacted] 36
Table 6. Maize Endogenous Elements in Plasmids and DP51291 Insertion 43
Table 7. SbS Junction Reads for DP51291 Maize, Control Maize, and Positive Controls....44
Table 8. Description of DNA Probes Used for Southern Hybridization....56
Table 9. Predicted and Observed Hybridization Bands on Southern Blots; [redacted] Digest 57
Table 10. Generations and Comparators Used for Analysis of DP51291 Maize....67
Table 11. Summary of Genotypic and Phenotypic Segregation Results for Five Generations of DP51291 Maize 68
Table 12. Identified Tryptic Peptides of DP51291 Maize-Derived IPD072Aa Protein Using LC MS Analysis....76
Table 13. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived IPD072Aa Protein Using LC-MS Analysis 76
Table 14. N-Terminal Amino Acid Sequence Analysis of the IPD072Aa Protein....77
Table 15. Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived PAT Protein Using LC-MS Analysis....84
Table 16. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived PAT Protein Using LC-MS Analysis....84
Table 17. N-Terminal Amino Acid Sequence Analysis of DP51291 Maize-Derived PAT Protein....85
Table 18. Identified Tryptic Peptides of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis....92
Table 19. Identified Chymotryptic Peptides of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis 92
Table 20. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis 94
Table 21. Across-Site Summary of IPD072Aa Protein Concentrations in DP51291 Maize 96
Table 22. Across-Site Summary of PAT Protein Concentrations in DP51291 Maize....96
Table 23. Across-Site Summary of PMI Protein Concentrations in DP51291 Maize....97
Table 24. Outcome of Across-Site Nutrient Composition Assessment for DP51291 Maize 100
Table 25. Proximates, Fiber, and Minerals Results for DP51291 Maize Forage 104
Table 26. Proximates and Fiber Results for DP51291 Maize Grain....106
Table 27. Fatty Acid Results for DP51291 Maize Grain....109
Table 28. Amino Acid Results for DP51291 Maize Grain 111
Table 29. Mineral Results for DP51291 Maize Grain 115
Table 30. Vitamin Results for DP51291 Maize Grain 117
Table 31. Secondary Metabolite and Anti-Nutrient Results for DP51291 Maize Grain....119
Table G1. Maize Growth Stage Descriptions 162
Table I1. Methods for Compositional Analysis 167
Table I2. Number of Sample Values Below the Lower Limit of Quantification (sprayed) 173

LIST OF FIGURES

Figure 1. Map of Plasmid 20
Figure 2. Map of the T-DNA Region from Plasmid 21
Figure 3. Map of Plasmid 22
Figure 4. Map of Plasmid 23
Figure 5. Map of Plasmid 24
Figure 6. Map of Plasmid 25
Figure 7. Map of Plasmid 26
Figure 8. Map of the Recombination Fragment Region from Plasmid 27
Figure 9. Map of Plasmid 28
Figure 10. Map of the T-DNA Region from Plasmid 29
Figure 11. Map of the DP51291 Maize Insertion 30
Figure 12. Map of the insertions in the DP51291 maize and DP23211 maize 31
Figure 13. Map of the Insertion in DP51291 Maize 45
Figure 14. SbS Results for Control Maize 47
Figure 15. SbS Results for Positive Control Samples 50
Figure 16. SbS Results for DP51291 Maize (Plant ID 434578663) 52
Figure 17. SbS Results for Representative Null Segregant Plant (Plant ID 434578664) 55
Figure 18. Map of Plasmid for Southern Analysis 57
Figure 19. Map of the Recombination Fragment Region from Plasmid for Southern Analysis 58
Figure 20. Map of the DP51291 Insertion for Southern Analysis 59
Figure 21. Southern Blot Analysis of of DP51291 Maize; Digest with *pmi* Probe 60
Figure 22. Southern Blot Analysis of DP51291 Maize; Digest with *mo-pat* Probe 61
Figure 23. Southern Blot Analysis of DP51291 Maize; Digest with *ipd072Aa* Probe 62
Figure 24. Event Development Process of DP51291 Maize 66
Figure 25. Breeding Diagram for DP51291 Maize and Generations Used for Analysis 67
Figure 26. Alignment of the Deduced Amino Acid Sequence of the IPD072Aa Protein 70
Figure 27. SDS-PAGE Analysis of the IPD072Aa Protein 72
Figure 28. Western Blot Analysis of the IPD072Aa Protein 73
Figure 29. Glycosylation Analysis of DP51291 Maize-Derived IPD072Aa Protein 75
Figure 30. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DP51291 Maize-Derived IPD072Aa Protein Using LC-MS Analysis 76
Figure 31. Alignment of the Deduced Amino Acid Sequence of PAT Protein Encoded by *pat* and *mo-pat* Genes 79
Figure 32. SDS-PAGE Analysis of DP51291 Maize-Derived PAT Protein 80
Figure 33. Western Blot Analysis of DP51291 Maize-Derived PAT 81
Figure 34. Glycosylation Analysis of DP51291 Maize-Derived PAT Protein 82
Figure 35. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DP51291 Maize-Derived PAT Protein Using LC-MS Analysis 85
Figure 36. Deduced Amino Acid Sequence of the PMI Protein 87
Figure 37. SDS-PAGE Analysis of DP51291 Maize-Derived PMI Protein 88
Figure 38. Western Blot Analysis of DP51291 Maize-Derived PMI Protein 89
Figure 39. Glycosylation Analysis of DP51291 Maize-Derived PMI Protein 90
Figure 40. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis 94
Figure A1. SbS Results for Plant ID 434578667 – DP51291 Maize (Transgenic) 138

Figure A2. SbS Results for Plant ID 434578672 – DP51291 Maize (Transgenic) 141
Figure A3. SbS Results for Plant ID 434578673 – DP51291 Maize (Transgenic) 144
Figure A4. SbS Results for Plant ID 434578675 – DP51291 Maize (Transgenic) 147

CHECKLISTS

		General requirements (3.1.1)
Check	**Page No.**	**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*
☑	11	B Applicant details
☑	11	C Purpose of the application
☑	12	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*
☑	10	K Statutory Declaration
☑	8	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*

Foods produced using gene technology (3.5.1)		
Check	**Page No.**	**Mandatory requirements**
☑	14	A.1 Nature and identity
☑	15	A.2 History of use of host and donor organisms
☑	17	A.3 Nature of genetic modification
☑	70	B.1 Characterisation and safety assessment
☑	70	B.2 New proteins
☑	95	B.3 Other (non-protein) new substances
☑	95	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
☑	95	B.5 Compositional analyses
☑	98	C Nutritional impact of GM food
☑	122	D Other information

STATUTORY DECLARATION

STATUTORY DECLARATION

Statutory Declarations Act 1959 [1]

I, [redacted] Regulatory Manager of Corteva Agriscience, Level 9, 67 Albert Ave, Chatswood, NSW 2067 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.

[*Signature of person making the declaration*]

Declared at Chatswood on 9th of February 2022

[1] http://www.comlaw.gov.au/Series/C1959A00052.

GENERAL INFORMATION ON THE APPLICATION

The chapter numbering follows section numbers from the FSANZ Application Handbook (Chapters 3.1 and 3.5.1).

B. APPLICANT

This

C. PURPOSE OF THE APPLICATION

Corteva Agriscience Pty Ltd, member of Corteva Agriscience group of companies (herein referred to as Corteva Agriscience), has developed DP51291 maize (OECD Unique Identifier DP- Ø51291-2), a new event that has been transformed to result in a plant with a single genetic construct to express the IPD072Aa, PAT and PMI proteins.

As a result of this application, Corteva Agriscience seeks an amendment of Standard 1.5.2 *Food produced using gene technology* by inserting the following into table to Schedule 26 3(4) after the last entry: *herbicide-tolerant and insect-protected corn line DP51291*.

D. JUSTIFICATION FOR THE APPLICATION

D(a) Need for the proposed change

Corteva Agriscience is a member of Excellence Through Stewardship™ (ETS). Corteva Agriscience has developed the new maize line DP51291, which will be commercialized in accordance with the ETS Product Launch Stewardship Guidance and in compliance with Corteva polices regarding stewardship of GM products. In line with these guidelines, Corteva's process for launches of new products includes a longstanding process to evaluate export market information, value chain consultations, and regulatory functionality, including obtaining regulatory clearance in applicable importing countries.

D(b) Advantage of the genetically modified food

DP51291 maize was genetically modified to express the IPD072Aa protein for control of susceptible corn rootworm (CRW) pests, the phosphinothricin acetyltransferase (PAT) protein for tolerance to glufosinate herbicide, and the phosphomannose isomerase (PMI) protein that was used as a selectable marker.

Maize has multiple downstream uses for feed, fuel, and food that are significant for the global supply of this crop commodity. The introduction of insect-resistant and herbicide-tolerant DP51291 maize is intended to help growers keep pace with increasing maize demand globally. The United States is one of the world's largest maize producers and a leading exporter of maize. In 2020, more than 14 billion bushels of maize were produced in the United States from approximately 90 million planted acres, valued at nearly $60 billion (NCGA, 2020; USDA-NASS, 2020). One of the most serious pests of maize in the United States is Western corn rootworm (WCR; *Diabrotica virgifera virgifera*), with economic losses of greater than $1 billion annually from both management costs and yield loss (Metcalf, 1986; PHI, 2010; Shrestha et al., 2018).

WCR damage has historically been managed with crop rotation, broad-spectrum soil insecticides, and transgenic crops expressing crystalline (Cry) proteins, such as Cry3 and Cry34/35 classes of proteins, developed from *Bacillus thuringiensis* (*Bt*). As adoption of *Bt* maize has increased, the selection pressure on target insects to develop resistance has become greater (Cullen et al., 2013). Insect resistance to transgenic traits can pose a threat to the long-term durability of *Bt* crops. As reduced performance of Cry3 and Cry34/35 proteins in maize has been reported in the scientific literature (Gassmann et al., 2016; Jakka et al., 2016), differentiated modes of action (MOA) are important for maintaining sustainable and durable CRW management (Gassmann et al., 2016; Niu et al., 2017). The IPD072Aa protein expressed in DP51291 maize has been demonstrated to be efficacious against susceptible CRW pests, including WCR, and provides a MOA that is separate and distinct from the currently available *Bt* protein-based MOAs for CRW control. DP51291 maize provides farmers with an additional control option for CRW pests to protect maize grain yield.

D.1 Regulatory impact

Corteva Agriscience has developed the new maize line DP51291, which will be commercialized in accordance with the ETS Product Launch Stewardship Guidance and in compliance with Corteva polices regarding stewardship of GM products. In line with these guidelines, Corteva's product launch process for launches of new products includes a longstanding process to evaluate export market information, value chain consultations, and regulatory functionality. Growers and end-users must take all steps within their control to follow appropriate stewardship requirements and confirm their buyer's acceptance of the grain or other material being purchased.

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% of 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. 440,000 t) 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).

D.1.1 Costs and Benefits for Industry, Consumers and Government

Corteva Agriscience acknowledges that the proposed amendment to the Standard will likely result in a time-limited exclusive capturable commercial benefit from the sale of seed in markets where DP51291 maize is to be cultivated 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 permits the import and use of food derived or developed from DP51291 maize.

D.1.2 Impact on international trade

The addition of DP51291 maize to Schedule 26 is anticipated to facilitate imports of maize from the applicable cultivation countries. Without such an approval, grain handlers would likely undertake a scientifically unnecessary and costly activities to segregate DP51291 maize and food products derived from it. Therefore, amending the Food Code to include DP51291 maize is anticipated to have a positive impact on Australian access to international commodity trade markets.

A TECHNICAL INFORMATION ON THE FOOD PRODUCED USING GENE TECHNOLOGY

A.1 NATURE AND IDENTITY OF THE GENETICALLY MODIFIED FOOD

A.1(a) Description of the GM organisms, nature and purpose of the genetic modification

DP51291 maize was genetically modified to produce the IPD072Aa protein for protection against susceptible corn rootworm (CRW) pests, the phosphinothricin acetyltransferase (PAT) protein for tolerance to the herbicidal active ingredient glufosinate-ammonium, and the phosphomannose isomerase (PMI) protein that was used as a selectable marker.

The three proteins (IPD072Aa, PAT and PMI) expressed by genetically modified DP51291 maize are familiar to Food Standards Australia New Zealand (FSANZ) as they are identical to the proteins that were recently assessed and approved in Australia New Zealand as part of insect resistant and herbicide tolerance corn line DP23211 for food release (Application A1202).

The IPD072Aa protein, encoded by the *ipd072Aa* gene, confers control of susceptible CRW pests when expressed in plants by causing disruption of the midgut epithelium. The *ipd072Aa* gene was identified and cloned from a *Pseudomonas chlororaphis* strain that was cultured from a soil sample (Schellenberger et al., 2016). The IPD072Aa protein and *ipd072Aa* gene are identical to that previously authorized as part of corn line DP23211 for food release (Application A1202).

The PAT protein, encoded by a maize-optimized version of the phosphinothricin acetyltransferase (*mo-pat*) gene from *Streptomyces viridochromogenes*, confers tolerance to the herbicidal active ingredient glufosinate-ammonium at current labeled rates by acetylating phosphinothricin, the active component of glufosinate-ammonium herbicide, to an inactive form. The PAT protein present in DP51291 maize is identical to the corresponding protein found in a number of approved events across several different crops that are currently in commercial use (CERA - ILSI Research Foundation, 2016; CERA, 2011; Hérouet et al., 2005).

The phosphomannose isomerase (PMI) protein is encoded by the *pmi* gene from *Escherichia coli*. The expressed PMI protein in plant tissue serves as a selectable marker during transformation which allows for tissue growth using mannose as the carbon source. The PMI protein present in DP51291 maize is identical to the corresponding protein found in a number of approved events across several different crops that are currently in commercial use (Negrotto et al., 2000).

A.1(b) GM organism identification

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants", this event has an OECD identifier of DP-Ø51291-2, also referred to as DP51291 maize.

A.1(c) Trade name

Maize event DP-Ø51291-2 is at a pre-commercialization stage and has not yet been assigned a commercial product name.

A.2 HISTORY OF USE OF THE HOST AND DONOR ORGANISMS

A.2(a) Donor organisms

Pseudomonas chlororaphis: donor of the ipd072Aa gene

- Class: Gammaproteobacteria
- Order: Pseudomonadales
- Family: Pseudomonadaceae
- Genus: *Pseudomonas*
- Species: *P. chloroaphis*
- Strain: SS143D5

The *ipd072Aa* gene is derived from *Pseudomonas chlororaphis*, a rod-shaped, aerobic, Gram-negative bacterium that is ubiquitous in soil. There is no known pathogenicity, toxicity or allergenicity of *Pseudomonas chlororaphis*. *P. chlororaphis* has a history of safe use as a biopesticide in the United States and Europe and has not been shown to be pathogenic to plants, livestock, and humans (Anderson et al., 2018). *P. choloraphis,* strain SS143D5 is the same donor of the *ipd072Aa* gene as used in previously assessed and authorized corn line DP23211.

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 Gram-positive, saprophytic, aerobic bacterium commonly found in soil. *S. viridochromogenes* is not considered pathogenic to humans or animals and is not known to be an allergen or toxin. *S. viridochromogenes* produces the tripeptide L-phosphinothricyl-L-alanyl-alanine (L-PPT), which was developed as a non-selective herbicide (OECD, 1999). *S. viridochromogenes*, strain Tü494 is the same donor of the *mo-pat* gene as used in previously assessed and authorized corn line DP23211.

Escherichia coli: donor of the pmi gene

- Class: Gammaproteobacteria
- Order: Enterobacteriales
- Family: Enterobacteriaceae
- Genus: *Escherichia*
- Species: *E. coli*
- Strain: K-12

Escherichia coli (*E. coli*) is a Gram-negative, anaerobic, rod-shaped bacterium. The strain *E. coli* K-12 is a strain which has been debilitated, does not normally colonize the human intestine and has a poor survival rate in the

environment. *E. coli* K-12 has a history of safe use in human drug and specialty chemical production (US-EPA, 1997). *E. coli* K-12 is the same donor of the *pmi* gene as used in previously assessed and authorized corn line DP23211.

Please refer to Section *B.2 New proteins* of this dossier for information relating to the potential allergenicity and toxicity of the expressed protein.

A.2(b) Host organism

Information relating to maize, the host organism, was included in previous safety assessments prepared by FSANZ. Repeating it is not considered necessary in this submission.

A.3 THE NATURE OF THE GENETIC MODIFICATION

A.3(a) Transformation method

Figure 1. Map of Plasmid

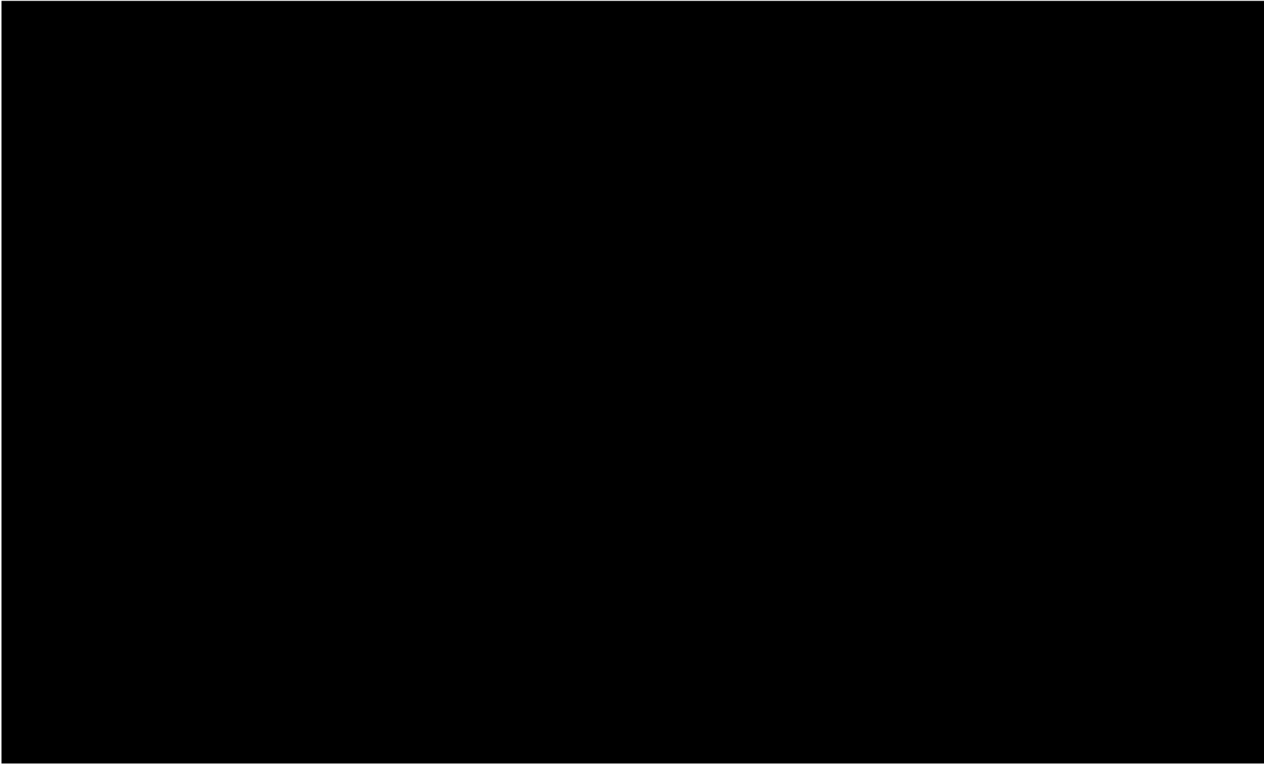


Figure 2. Map of the T-DNA Region from Plasmid

Figure 3. Map of Plasmid

Figure 4. Map of Plasmid

Figure 5. Map of Plasmid

Figure 6. Map of Plasmid

Figure 7. Map of Plasmid

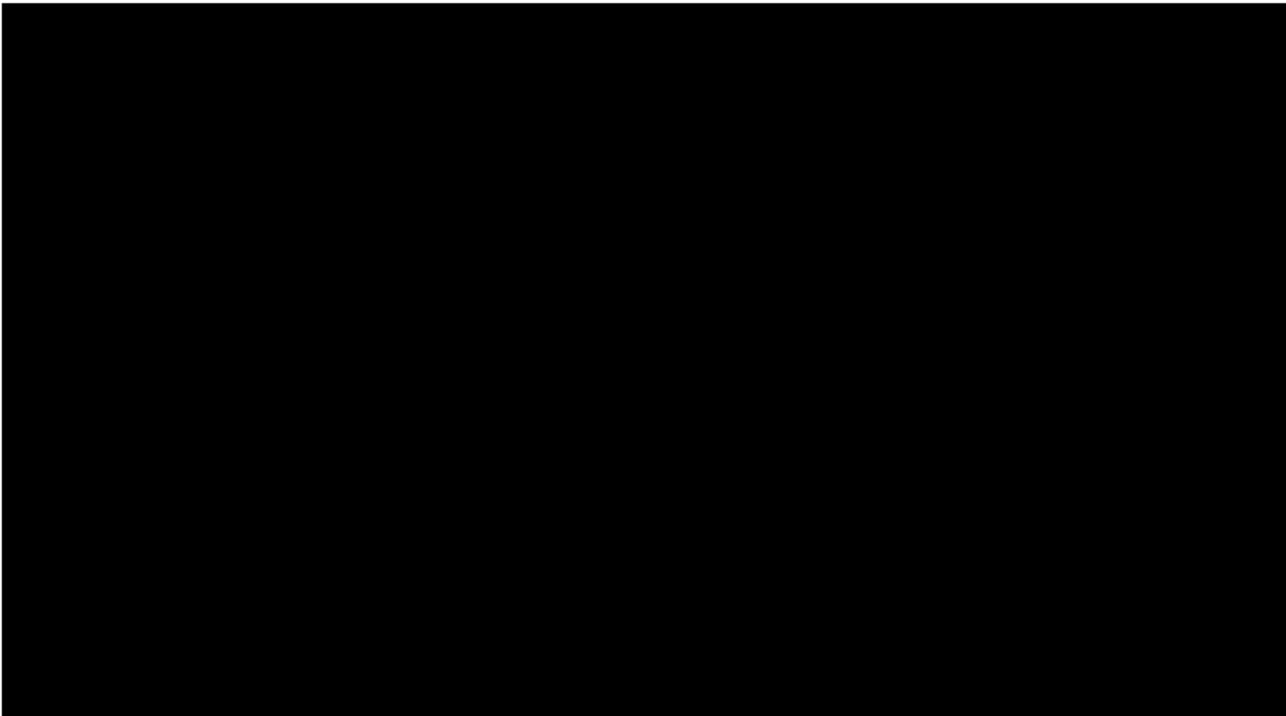


Figure 8. Map of the Recombination Fragment Region from Plasmid

Figure 9. Map of Plasmid

Figure 10. Map of the T-DNA Region from Plasmid

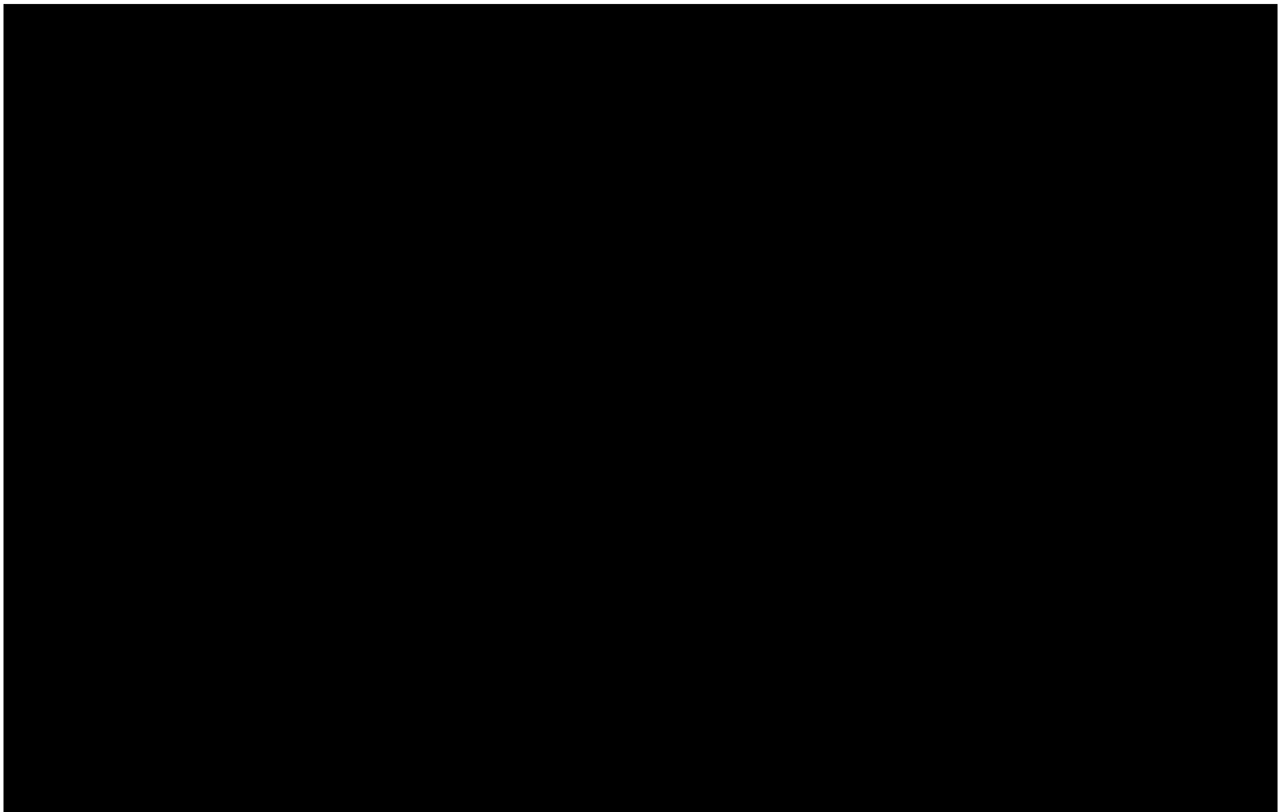


Figure 11. Map of the DP51291 Maize Insertion

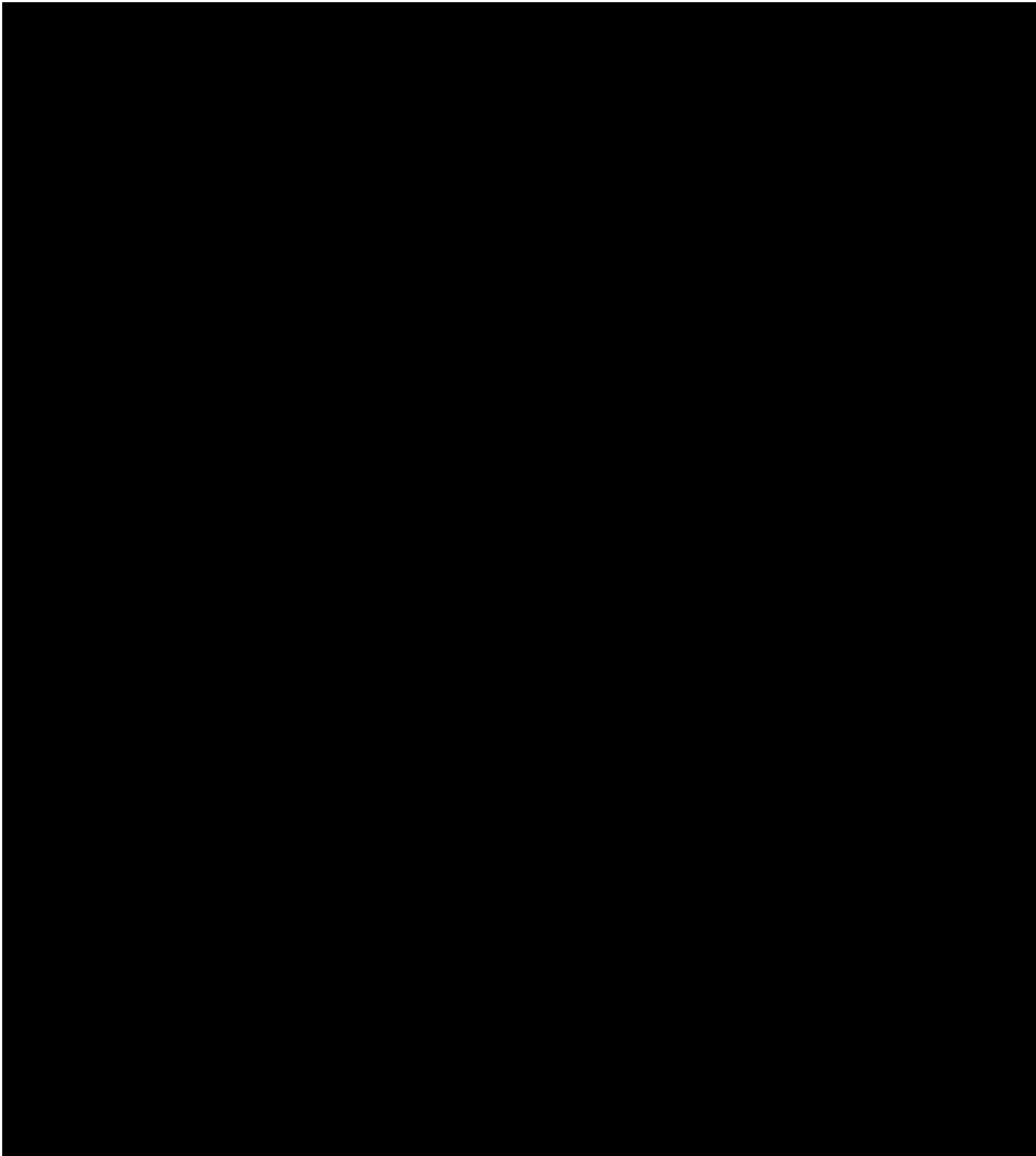


Figure 12. Map of the insertions in the DP51291 maize and DP23211 maize

(A) Schematic diagram of the insertion in the DP51291 maize genome. The DP51291 insert includes three cassettes (*ipd072Aa*, *mo-pat*, and *pmi*), which are identical to the corresponding gene cassettes inserted in the DP23211 maize genome.

(B) Schematic diagram of the insertion in the DP23211 maize genome. The DP23211 insert includes four cassettes (DvSSJ1 fragment cassette and *ipd072Aa*, *mo-pat*, and *pmi*), the DvSSJ1 fragment cassette is only present in the DP23211 maize insertion. The corn line DP23211 was approved by Food Standards Australia New Zealand (FSANZ) (Application A1202).

Description of the T-DNA Region of Plasmid

Table 4. Description of the Genetic Elements in Plasmid

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

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

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

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

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

A.3(b) Construct and the transformation vectors used

Please refer to Section *A.3(a)* *Transformation method* for the vectors used in transformation and to Table 4 for a description of the genetic elements of the Plasmid [redacted] Figure 9 for the map of Plasmid [redacted]; Table 5 for the description of the genetic elements in T-DNA Region of Plasmid [redacted]; and Figure 10 for the T-DNA region maps from Plasmid [redacted].

A.3(c) Molecular characterisation

Molecular characterization of DP51291 maize plants was conducted using Southern-by-Sequencing (SbS™ technology) to determine the insertion copy number and organization within the plant genome and to confirm the absence of plasmid backbone and other unintended plasmid sequences. Southern blot analysis was performed to confirm stable genetic inheritance of the inserted *ipd072Aa*, *pmi*, and *mo-pat* gene cassettes. Segregation analysis was conducted for five generations of DP51291 maize to confirm stable Mendelian inheritance. Sanger sequencing was conducted to determine the DNA sequence of the DP51291 insert and flanking genomic regions. Additionally, a bioinformatic assessment was conducted to evaluate the potentially-expressed peptides within an insertion or crossing the boundary between an insertion and its genomic borders for similarity to known and putative allergens and toxins.

Based on the SbS analysis described below, it was determined that a single copy of the inserted DNA with the expected organization (Figure 11), and that no additional insertions, plasmid backbone, or other unintended plasmid sequences are present in DP51291 maize. In addition, Southern blot analysis across five breeding generations confirmed the stable genetic inheritance of the DNA insertion in DP51291 maize. Segregation analysis across five breeding generations confirmed a stable Mendelian inheritance pattern. Sanger sequencing was conducted to determine the DNA sequence of the DP5291 insert and flanking genomic regions.

Southern-by-Sequencing (SbS) Analysis to Determine Insertion Copy Number and Organization and Confirm the Absence of Plasmid Backbone and Other Unintended Plasmid Sequences [redacted] 2022 (PHI-2022-120 study))

SbS analysis utilizes probe-based sequence capture, Next Generation Sequencing (NGS) techniques, and bioinformatics analysis 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 intended insertion map, linearized transformation plasmid maps, and the endogenous genomic reference, unique junctions due to inserted DNA are identified in the bioinformatics analysis. This information is used to determine the number and organization of insertions within the plant genome and confirm the absence of plasmid backbone and other unintended plasmid sequences.

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

the overall length of the multiple reads for that junction may vary due to the data analysis process. The number of unique junctions is related to the number of plasmid insertions present in the maize genome (for example, a single T-DNA insertion is expected to have two unique junctions). Detection of additional unique junctions beyond the two expected for a single insertion would indicate the presence of rearrangements or deletions within the insertion, or additional insertions containing plasmid DNA. The absence of any junctions indicates there are no detectable insertions within the maize genome.

The segregating T1 generation of DP51291 maize was analyzed by SbS, using capture probes targeting all sequences of the plasmids utilized to create DP51291 maize ( ) to determine the insertion copy number and organization and to confirm the absence of plasmid backbone or other unintended plasmid sequences. SbS was also performed on non-GM near-isoline PHR03 maize as a control, and on positive control samples of each plasmid spiked into non-GM near-isoline PHR03 maize DNA to confirm that the assay could reliably detect plasmid fragments within genomic DNA. Based on the results obtained for DP51291 maize, a schematic diagram of the DP51291 insertion was developed and is provided in Figure 13.

Several genetic elements in the plasmids used in the positive control samples are derived from maize, and thus the homologous elements in the maize genome will be captured by the full-coverage probes used in the SbS analysis. These endogenous elements ; Table 6 and Figure 14 to Figure 17) will have sequencing reads in the SbS results due to the homologous elements in the maize genome. However, if no junctions are detected, these sequencing reads only indicate the presence of the endogenous elements in their normal context of the maize genome and are not from inserted DNA.

SbS analysis results for the control maize are shown in Figure 14 and the positive control samples are presented in Figure 15. Results from the segregating T1 generation of DP51291 maize are presented in Figure 16 to Figure 17 and Appendix A - Figures A1 to A4.

SbS Analysis of the PHR03 Control Maize

Sequencing reads of the PHR03 control maize were aligned to the intended insertion and plasmid maps (Figure 13); however, coverage was obtained only for the endogenous genetic elements derived from the maize genome. These sequence reads were due to capture and sequencing of these genetic elements in their normal context within the PHR03 control maize genome (Table 6). Variation in coverage of the maize endogenous elements is due to sequence variations between the PHR03 control maize and the maize varieties from which the genetic elements in the plasmids were derived. No junctions were detected between plasmid sequences and the maize genome (Table 7), indicating that there are no plasmid DNA insertions in the control maize, and the sequence reads were solely due to the endogenous genetic elements present in the PHR03 control maize genome.

SbS Analysis of the Positive Control Samples Containing Spiked-in Plasmid DNA

SbS analysis of the positive control samples resulted in sequence coverage across the entire length of each plasmid (Figure 15), indicating that the SbS assay utilizing the full-coverage probe library is sensitive enough to detect sequences. Junctions were identified in that were due to sequencing reads from one part of the plasmid aligning to identical or highly similar sequences located in different regions of the plasmid (Figure 15), but these junctions are artifacts of the alignment and do not indicate plasmid insertions in the maize genome. Junctions were also identified in ; these were determined to be due to contamination of the sample by DNA sequences that are similar but not identical to sequences in and they do not indicate insertions in the maize genome.

SbS Analysis of the T1 Generation of DP51291 Maize

SbS analysis of the segregating T1 generation of DP51291 maize showed five positive plants that contained the inserted DNA (Table 7; Figure 16; and Appendix A - Figures A1 to A4). Each of these plants contained two unique genome-insertion junctions, one at each end of the insertion; the two unique junctions were identical across the five plants. The insertion, derived from , is bp in length, starting with the 5′ junction at bp of the intended insertion and ending with the 3′ junction at bp (Figure 13), indicating that the last three bp of the and the first bp of the from were incorporated into the DP51291 maize insertion as part of the landing pad. The number of sequencing reads at the 5′ and 3′ junctions is provided in Table 7. There were no other junctions between the plasmid sequences and the maize genome detected in the plants, indicating that there is no additional plasmid-derived insertions present in DP51291 maize.

Alignments of the reads from the five positive plants to the seven plasmid maps (Figure 16 and Appendix A - Figures A1 to A4) show coverage of the genetic elements found in the intended insertion, along with coverage of the endogenous elements in the plasmids that were not incorporated into the insertion (. Reads also aligned to the and terminator elements, sites, and other sequences located outside of the intended insertion regions in although these sequences were not incorporated into the insertion. The NGS reads that aligned to these sequences on the plasmid maps are due to regions from the insertion that contain identical sequences, but do not indicate incorporation of the sequences from unintended sources as there are no additional junctions. There were no unexpected junctions between non-contiguous regions of the intended insertion identified, indicating that there are no rearrangements, deletions, or duplications in the inserted DNA. Furthermore, there were no junctions between the sequences that were not intended to be incorporated from any of the plasmids involved in the production of DP51291 maize and maize genome sequences, demonstrating that no plasmid backbone or other unintended plasmid sequences were incorporated into DP51291 maize.

Each of the five null segregant plants from the T1 generation of DP51291 maize that was negative for the DP51291 insertion yielded sequencing reads for the endogenous genetic elements derived from the maize genome (a representative plant is presented in Figure 17). There were no junctions between plasmid sequences and the maize genome detected in these plants, indicating that these plants did not contain any insertions derived from

SbS analysis of the T1 generation of DP51291 maize demonstrated that DP51291 maize contains a single copy of the inserted DNA derived from , with the expected organization, and that no additional insertions, plasmid backbone, or other unintended plasmid sequences are present in its genome.

Additional details regarding analytical methods for SbS analysis are provided in Appendix A.

Table 6. Maize Endogenous Elements in Plasmids and DP51291 Insertion

Number[a]	Name of Endogenous Element[b]	Present in Plasmid(s) or Insertion

[a] The numbers indicating endogenous genetic elements are shown as circled numbers found below linear construct maps in Figure 14and Figure 15 and Appendix A - Figures A1-A4.
[b] As shown in the plasmid, T-DNA, and recombination fragment maps in Figure 1, Figure 3 to Figure 8, and the intended insertion map in Figure 11.

Table 7. SbS Junction Reads for DP51291 Maize, Control Maize, and Positive Controls

Sample Description	Total Reads at 5' Genomic Junction[a]	Unique Reads at 5' Genomic Junction[b]	Total Reads at 3' Genomic Junction[c]	Unique Reads at 3' Genomic Junction[d]	DP51291 Insertion
T1 Generation Plant ID 434578663	210	64	47	19	+
T1 Generation Plant ID 434578664	0	0	0	0	-
T1 Generation Plant ID 434578665	0	0	0	0	-
T1 Generation Plant ID 434578666	0	0	0	0	-
T1 Generation Plant ID 434578667	250	78	65	27	+
T1 Generation Plant ID 434578669	0	0	0	0	-
T1 Generation Plant ID 434578672	150	48	54	27	+
T1 Generation Plant ID 434578673	155	46	36	20	+
T1 Generation Plant ID 434578675	211	73	61	27	+
T1 Generation Plant ID 434578677	0	0	0	0	-
PHR03 Control Maize	0	0	0	0	-
Positive Control	0	0	0	0	-
Positive Control	0	0	0	0	-
Positive Control	0	0	0	0	-
Positive Control	0	0	0	0	-
Positive Control	0	0	0	0	-
Positive Control	0	0	0	0	-
Positive Control	0	0	0	0	-

[a] Total number of sequencing reads across the 5' junction of the DP51291 insertion.
[b] Unique sequencing reads establishing the location of the 5' genomic junction of the DP51291 insert (Figure 13). Multiple identical NGS reads are condensed into each unique read.
[c] Total number of sequencing reads across the 3' junction of the DP51291 insertion.
[d] Unique sequencing reads establishing the location of the 3' genomic junction of the DP51291 insert (Figure 13). Multiple identical NGS reads are condensed into each unique read.

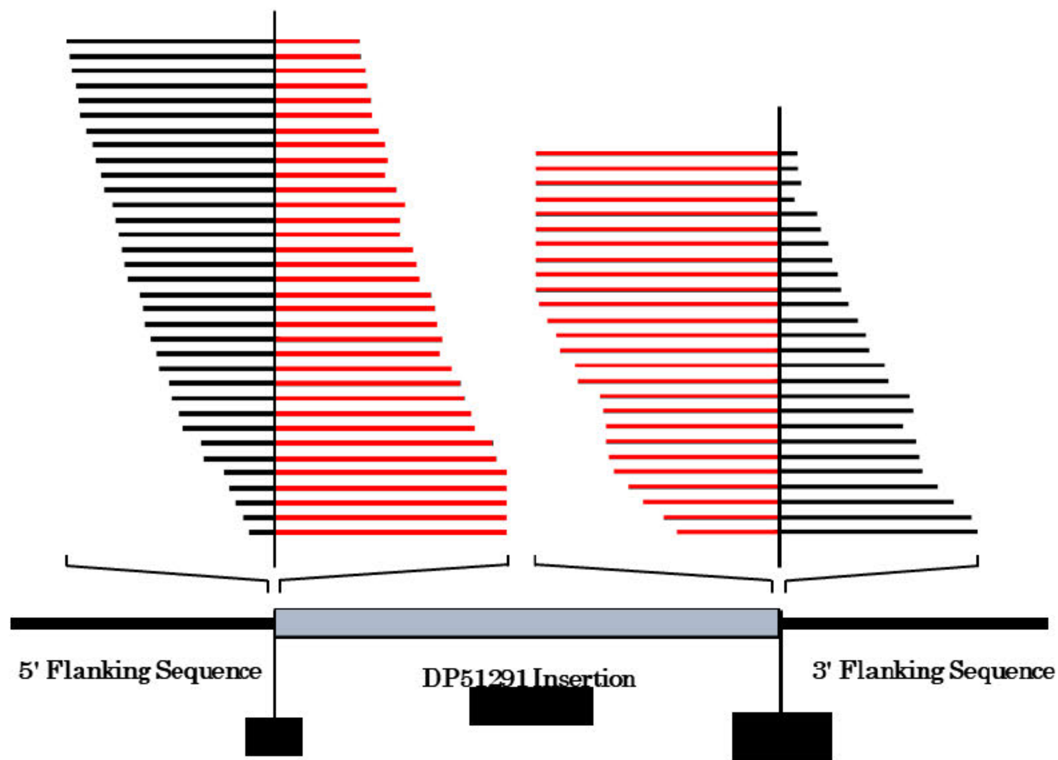


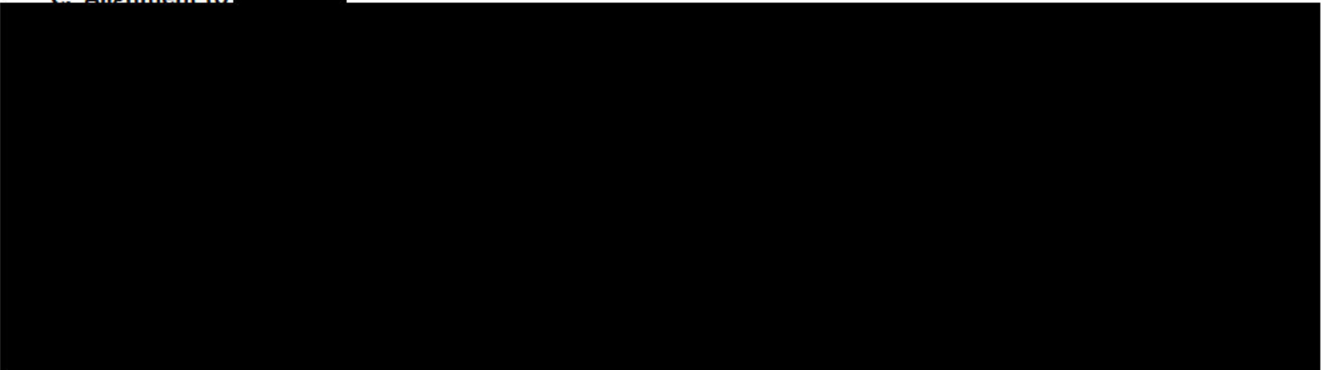
Figure 13. Map of the Insertion in DP51291 Maize

Schematic diagram of the DNA insertion in DP51291 maize based on the SbS analysis described. The flanking maize genomic regions are indicated in the map by black bars. A single copy of the insertion, derived from [redacted] and shown by the gray box, is integrated into the DP51291 maize genome. Vertical lines show the locations of the two unique genome-insertion junctions. The numbers below the map indicate the bp location of the junction nucleotide in reference to the sequence of the intended insertion. Representative individual sequencing reads across the junctions are shown as horizontally stacked lines above each junction (not to scale); black indicates genomic flanking sequence and red indicates inserted DNA sequence within each read.

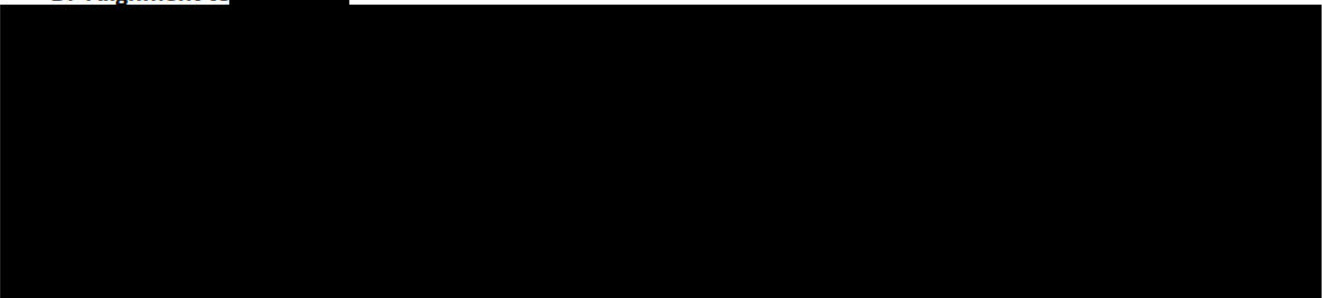
A. Alignment to Intended Insertion

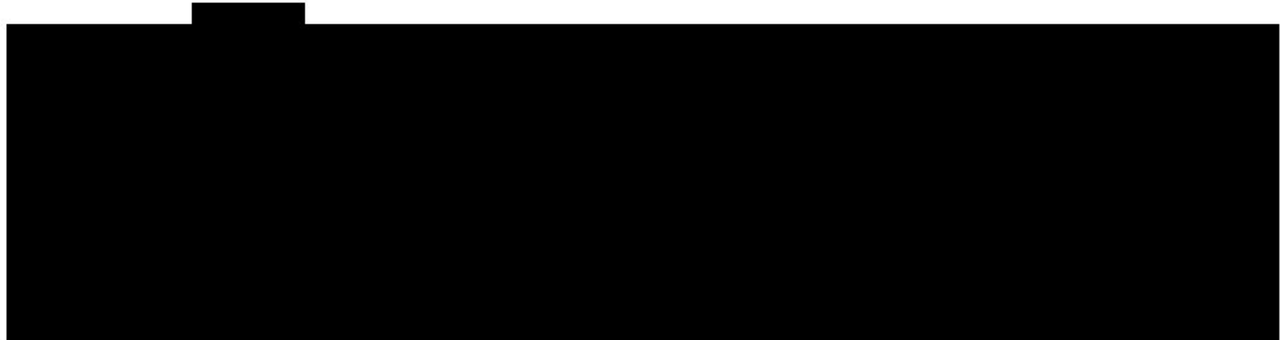
B. Alignment to

C. Alignment to

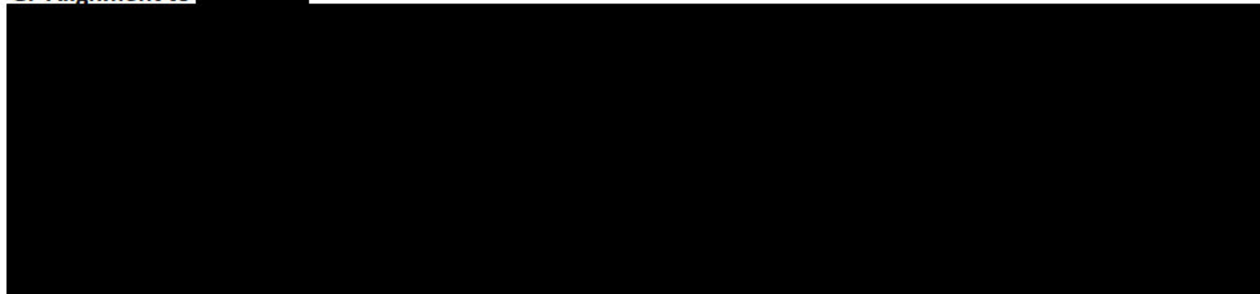


D. Alignment to





G. Alignment to



H. Alignment to

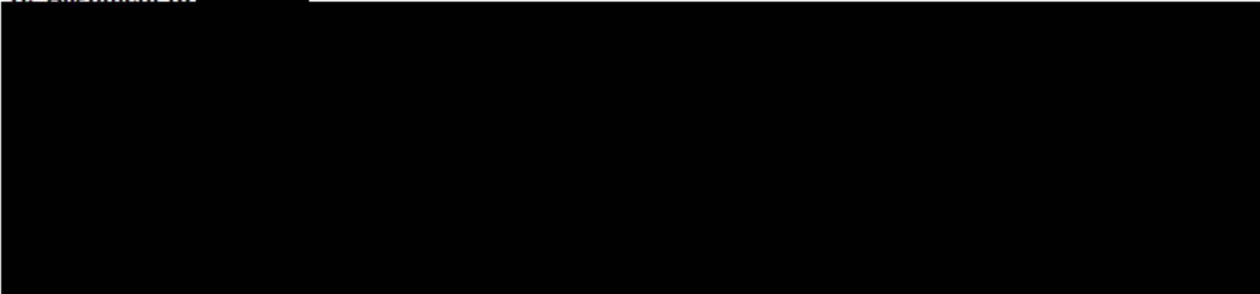


Figure 14. SbS Results for Control Maize

The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or plasmid backbone sequence present in the PHR03 control maize. FRT sites are indicated by red arrows. **A)** SbS results for control maize aligned against the intended insertion (bp; Figure 11). Coverage was obtained only for regions derived from maize endogenous elements. Variation in coverage of the endogenous elements is due to sequence variations between the control maize and the source of the corresponding genetic elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in the control maize, and the sequencing reads are solely due to the endogenous elements present in the PHR03 genome. **B)** SbS results aligned against the plasmid sequence bp; Figure 9). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid sequence (bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid sequence

bp; Figure 5). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid sequence (
bp; Figure 4). Coverage was obtained only for the endogenous elements. **F)** SbS results aligned against the plasmid sequence
bp; Figure 3). Coverage was obtained only for the endogenous elements. **G)** SbS results aligned against the plasmid sequence
bp; Figure 6). Coverage was obtained only for the endogenous elements. **H)** SbS results aligned against the plasmid sequence
bp; Figure 7). Coverage was obtained only for the endogenous elements.

A. Alignment to

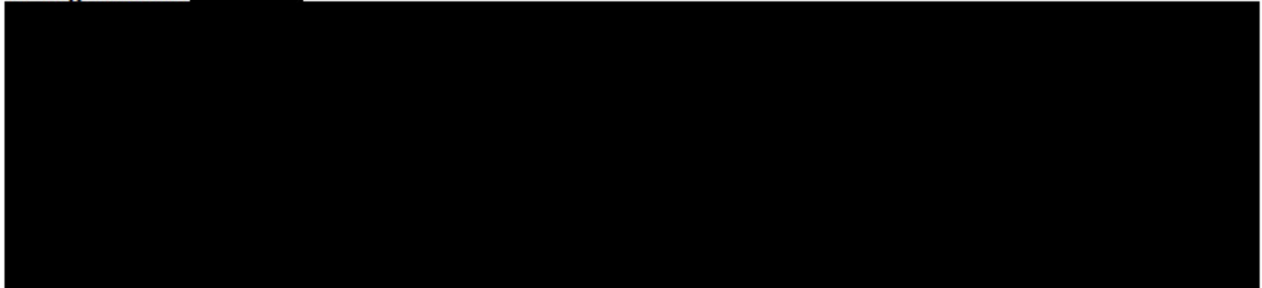
B. Alignment to

C. Alignment to

D. Alignment to

E. Alignment to

F. Alignment to



G. Alignment to

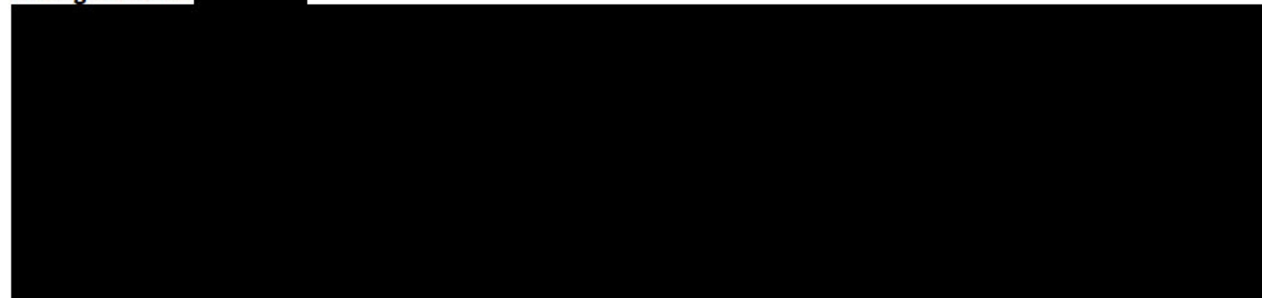


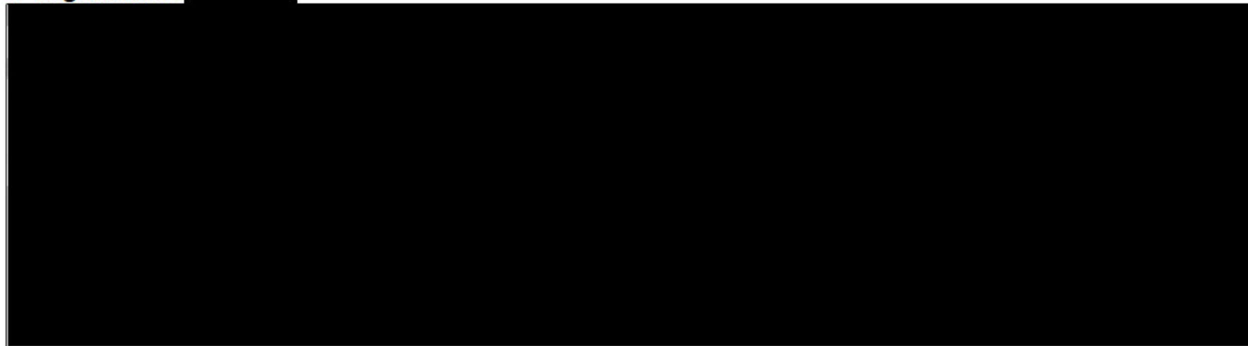
Figure 15. SbS Results for Positive Control Samples

The positive control samples consisted of separate control maize DNA samples individually spiked with each plasmid. The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in the plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. Junctions located at the left and right ends of the graphs are artifacts of mapping a circular plasmid to a linear map and show the start and end points of the plasmid sequence but do not indicate insertions in genomic DNA. **A)** SbS results of the positive control sample aligned against bp; Figure 9). Coverage was obtained across the full length of the plasmid, indicating successful capture of sequences by the SbS probe library. The junction located at bp is the result of reads from bp aligning to an identical -bp region of the plasmid at bp . **B)** SbS results of the positive control sample aligned against bp; Figure 1). Coverage was obtained across the full length of the plasmid, indicating successful capture of sequences by the SbS probe library. **C)** SbS results of the positive control sample aligned against (bp; Figure 5). Coverage was obtained across the full length of the plasmid, indicating successful capture of sequences by the SbS probe library. **D)** SbS results of the positive control sample aligned against bp; Figure 4). Coverage was obtained across the full length of the plasmid, indicating successful capture of sequences by the SbS probe library. **E)** SbS results of the positive control sample aligned against (bp; Figure 3). Coverage was obtained across the full length of the plasmid, indicating successful capture of sequences by the SbS probe library. The junction located at bp is the result of reads from the site at bp aligning to a nearly identical -bp region of the plasmid from the site at bp . **F)** SbS results of the positive control sample aligned against bp; Figure 6). Coverage was obtained across the full length of the plasmid, indicating successful capture of sequences by the SbS probe library. The junction located at bp is the result of reads from bp aligning to an identical but complementary -bp region of the plasmid at bp . **G)** SbS results of the positive control sample aligned against (bp; Figure 7). Coverage was obtained across the full length of the plasmid, indicating successful capture of sequences by the SbS probe library. The junctions at bp and are from reads that contain regions that align to ; however, the remaining portions of the reads don't align to either or the endogenous genomic reference and were determined to be due to contamination.

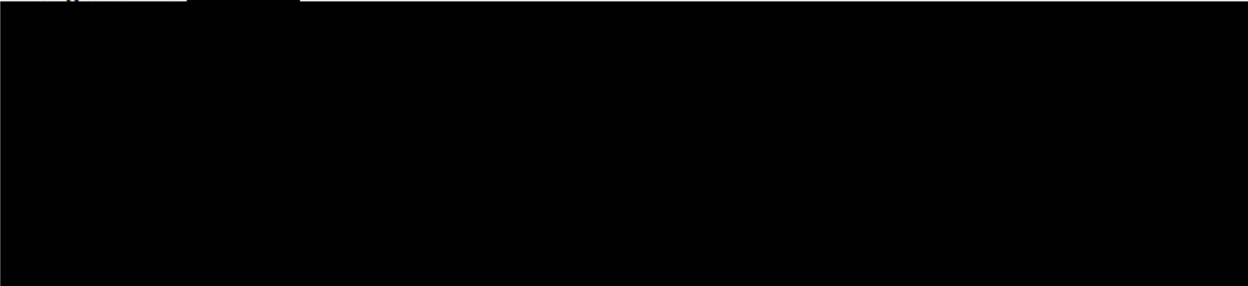
A. Alignment to Intended Insertion

B. Alignment to

C. Alignment to



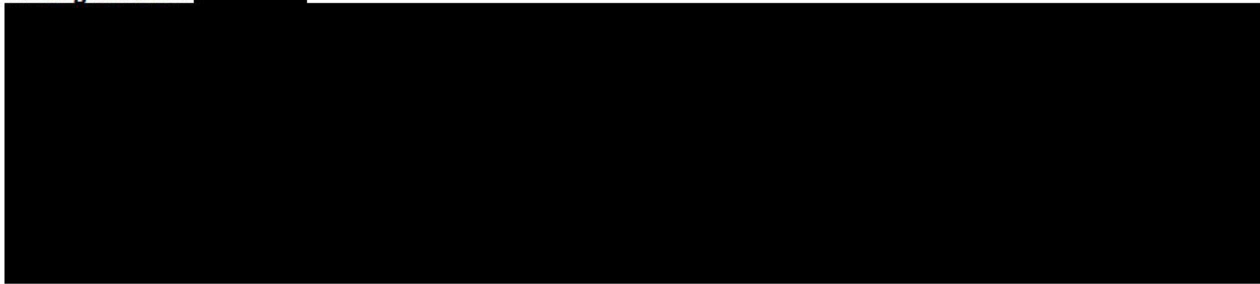
D. Alignment to



E. Alignment to

F. Alignment to

G. Alignment to



H. Alignment to

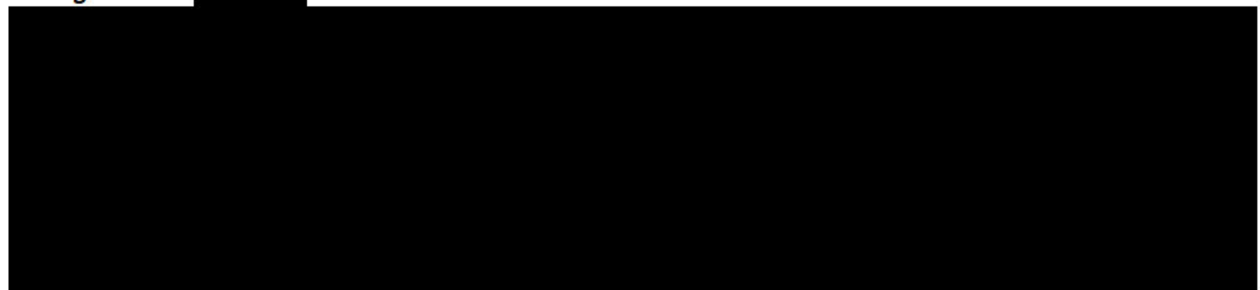


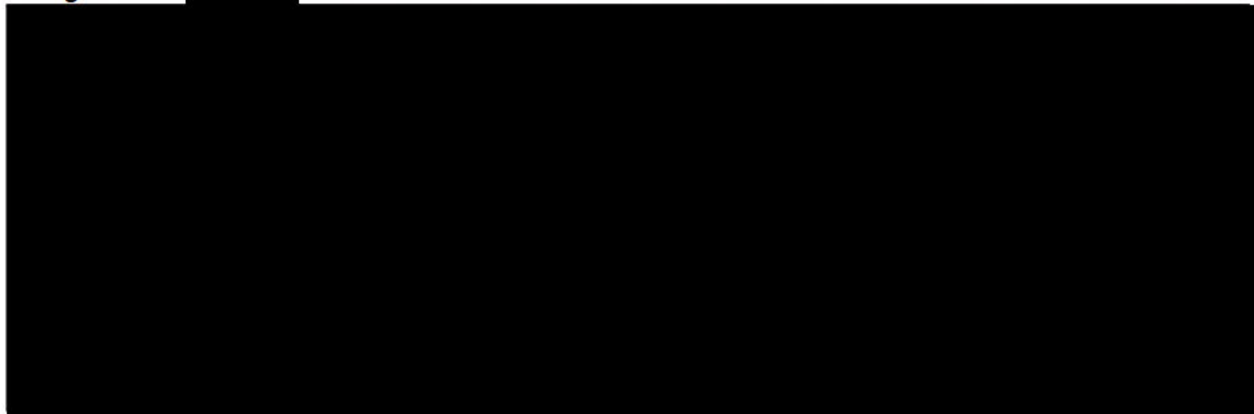
Figure 16. SbS Results for DP51291 Maize (Plant ID 434578663)

The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion bp; Figure 11), indicating that this plant contains the intended insertion. The bottom part of the graph shows the two plasmid-to-genome sequence junctions identified by SbS; the numbers refer to the bp location of each junction relative to the intended insertion (Figure 11). The presence of only two junctions demonstrates the presence of a single insertion in the DP51291 maize genome. **B)** SbS results aligned against the plasmid sequence bp; Figure 9). Coverage was obtained for the elements between transferred into DP51291 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region between the that were not transferred into the DP51291 maize genome, and to the terminator (*), terminator (†), and site (‡) elements outside of the FRT sites, due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in . Finally, coverage was obtained to the regions around the RB and LB elements that match the corresponding sequences derived from . **C)** SbS results aligned against the plasmid sequence bp; Figure 1). Coverage was obtained for the elements found in the intended insertion (between the to the first site,

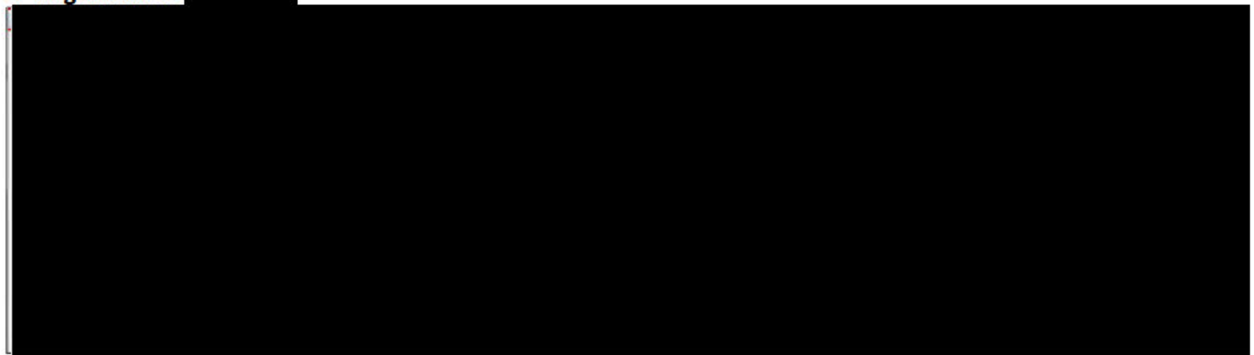
the second [redacted] to [redacted] and between [redacted] to the [redacted]), along with the endogenous [redacted] intron element, an [redacted] site (‡) and the cassettes between the FRT sites that are identical to those found in the final DP51291 maize insertion. **D)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 5). Coverage was obtained only for the endogenous elements along with the [redacted] terminator element (*). **E)** SbS results aligned against the plasmid [redacted] sequence ([redacted] bp; Figure 4). Coverage was obtained only for the endogenous elements along with the [redacted] terminator element (*). **F)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 3). Coverage was obtained for the endogenous elements along with the FRT sites, [redacted] terminator elements (*), [redacted] terminator (†), and [redacted] sites (‡) that are identical to sequences in the DP51291 maize insertion. **G)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 6). Coverage was obtained for the endogenous elements. The coverage at approximately [redacted] bp is due to a [redacted]-bp region that is an exact match to a region in the insertion derived from [redacted]. **H)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 7). Coverage was obtained for the endogenous elements along a [redacted]-bp region that is identical to the [redacted] element in the insertion derived from [redacted] (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or plasmid backbone or other unintended sequences present in DP51291 maize.

A. Alignment to Intended Insertion

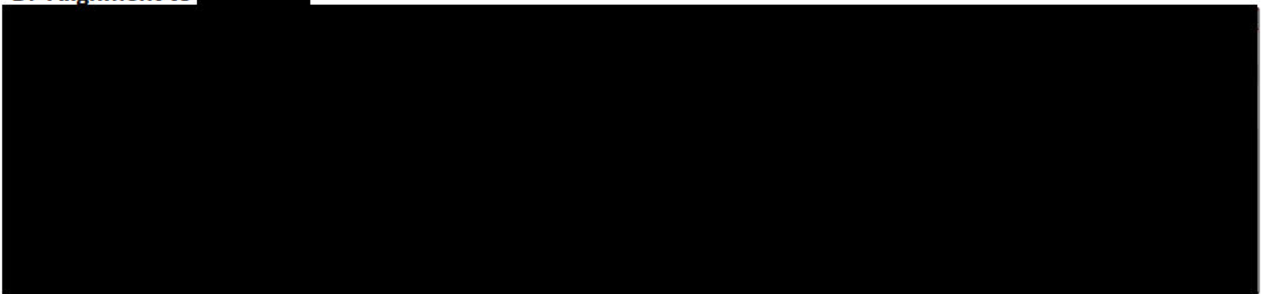
B. Alignment to



C. Alignment to



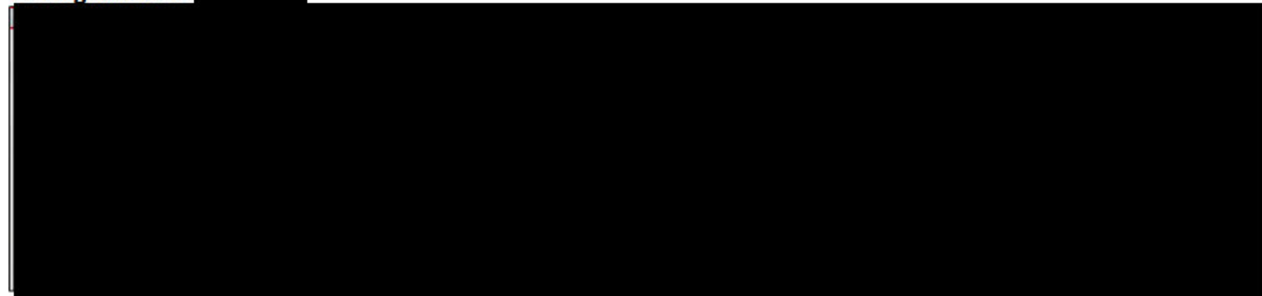
D. Alignment to



E. Alignment to

F. Alignment to

G. Alignment to



H. Alignment to

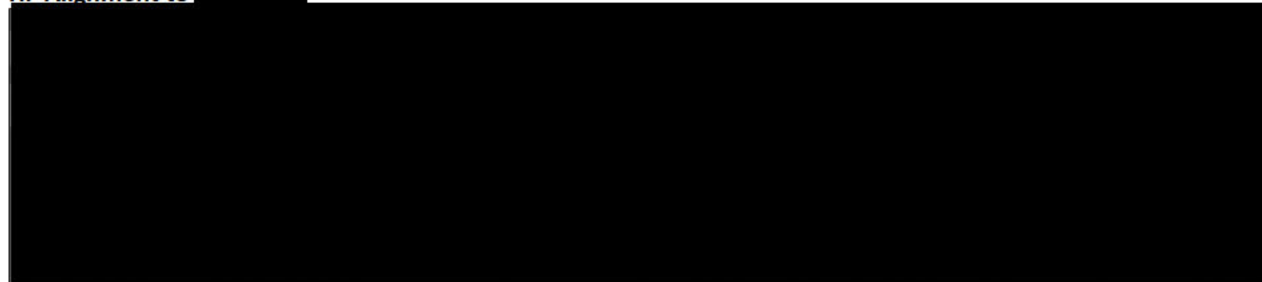


Figure 17. SbS Results for Representative Null Segregant Plant (Plant ID 434578664)

The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or plasmid backbone or other unintended sequences present in this plant. **A)** SbS results aligned against the intended insertion bp; Figure 11). Coverage was obtained only for regions derived from maize endogenous elements. As no junctions were detected between plasmid sequences and the maize genome, there are no DNA insertions identified in this plant, and the sequencing reads are solely due to the endogenous elements present in the PHR03 genome. **B)** SbS results aligned against the plasmid sequence bp; Figure 9). Coverage was obtained only for the endogenous elements. **C)** SbS results aligned against the plasmid sequence bp; Figure 1). Coverage was obtained only for the endogenous elements. **D)** SbS results aligned against the plasmid sequence bp; Figure 5). Coverage was obtained only for the endogenous elements. **E)** SbS results aligned against the plasmid sequence bp; Figure 4). Coverage was obtained only for the

endogenous elements. **F)** SbS results aligned against the plasmid sequence bp; Figure 3). Coverage was obtained only for the endogenous elements. **G)** SbS results aligned against the plasmid sequence bp; Figure 6). Coverage was obtained only for the endogenous elements. **H)** SbS results aligned against the plasmid sequence bp; Figure 7). Coverage was obtained only for the endogenous elements.

Southern Analysis to Determine Stable Genetic Inheritance (, 2022 (PHI-2022-064 study))

Southern blot analysis was performed on five generations of DP51291 maize to evaluate the stability of the inserted *ipd072Aa*, *mo-pat*, and *pmi* gene cassettes across multiple generations.

Restriction enzyme indicated in Figure 18 and Figure 19) was selected to verify the stability of the DP51291 maize insertion across the five generations (T1, T2, T3, T4, and T5) of DP51291 maize plants. was selected because there is a single restriction site within the DP51291 maize insertion, which provides a means to uniquely identify the event, as additional sites would be in the adjacent flanking genomic DNA (Figure 20). Genomic DNA samples extracted from leaf tissue of the five generations of DP51291 maize and control PHR03 maize plants were digested with and hybridized with the the *pmi, mo-pat* and *ipd072Aa* probes for Southern analysis. Hybridization patterns of these probes exhibited event-specific bands unique to the DP51291 maize insertion, and thus provided a means of verification that the genomic border regions of the DP51291 maize insertion were not changed across the five generations during breeding. Plasmid was added to control maize DNA, digested with and included on the blot to verify successful probe hybridization. The probes used for Southern hybridization are described in Table 8 and shown in Figure 18.

Hybridization of the *pmi* probe to digested genomic DNA resulted in a consistent band of approximately bp in all five generations of DP51291 maize (Table 9, Figure 21). This result confirmed that the 5' border fragment, containing the *pmi* gene in the DP51291 maize insertion, is intact and stable across the five generations of DP51291 maize. The positive control lanes containing plasmid DNA showed the expected band of bp, confirming successful hybridization of the *pmi* probe. No bands were observed in the control maize plant.

Hybridization of the *mo-pat* and *ipd072Aa* probes to digested genomic DNA resulted in a consistent band of approximately bp in all five generations of DP51291 maize analyzed (Table 9, Figure 22 and Figure 23, respectively). These results confirmed that the 3' border fragment, containing the *mo-pat* and *ipd072Aa* genes in the DP51291 maize insertion, is intact and stable across the five generations of DP51291 maize. The positive control lanes containing plasmid DNA showed the expected band of bp, confirming successful hybridization of the *mo-pat* and *ipd072Aa* probes. No bands were observed in the control maize plant.

The presence of equivalent bands from hybridization with each of the *ipd072Aa*, *mo-pat*, and *pmi* probes within the plants from all five generations analyzed confirms that the inserted DNA in DP51291 maize is consistent and stable across multiple generations during the breeding process.

Additional details regarding analytical methods for Southern analysis are provided in Appendix B.

Table 8. Description of DNA Probes Used for Southern Hybridization

Genetic Element/ Probe Name	Probe Length (bp)	Position on Plasmid (bp to bp)[a]	Position on DP51291 Insertion Map (bp to bp)[b]
pmi[c]			
mo-pat			
ipd072Aa			

[a] The probe position is based on the plasmid map (Figure 18).

[b] The probe position is based on the DP51291 insertion map (Figure 20).

[c] The probe comprises two fragments that are combined in a single hybridization solution.

Table 9. Predicted and Observed Hybridization Bands on Southern Blots; [illegible] Digest

Probe Name	Predicted and Observed Fragment Size from Plasmid [illegible] (bp)[a]	Predicted Fragment Size from DP51291 Insertion Map (bp)[b]	Observed Fragment Size in DP51291 Maize (bp)[c]	Figure
pmi	[illegible]	[illegible]	[illegible]	Figure 21
mo-pat	[illegible]	[illegible]	[illegible]	Figure 22
ipd072Aa	[illegible]	[illegible]	[illegible]	Figure 23

[a] Predicted and observed fragment sizes based on the [illegible] plasmid map (Figure 18).
[b] Predicted sizes based on the DP51291 insertion map (Figure 20).
[c] Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker III and VII fragments on the Southern blots. Due to inability to determine the exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.

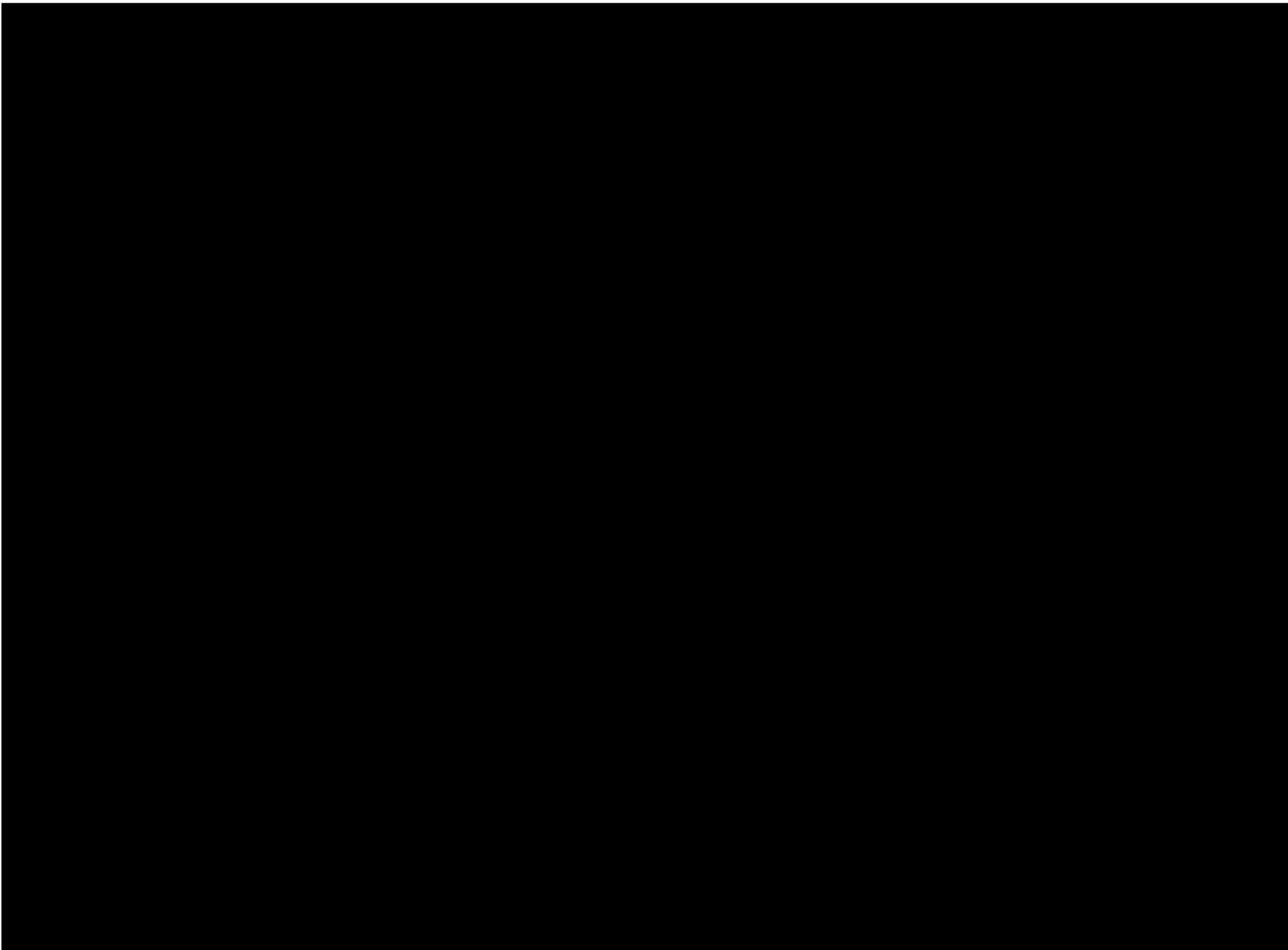
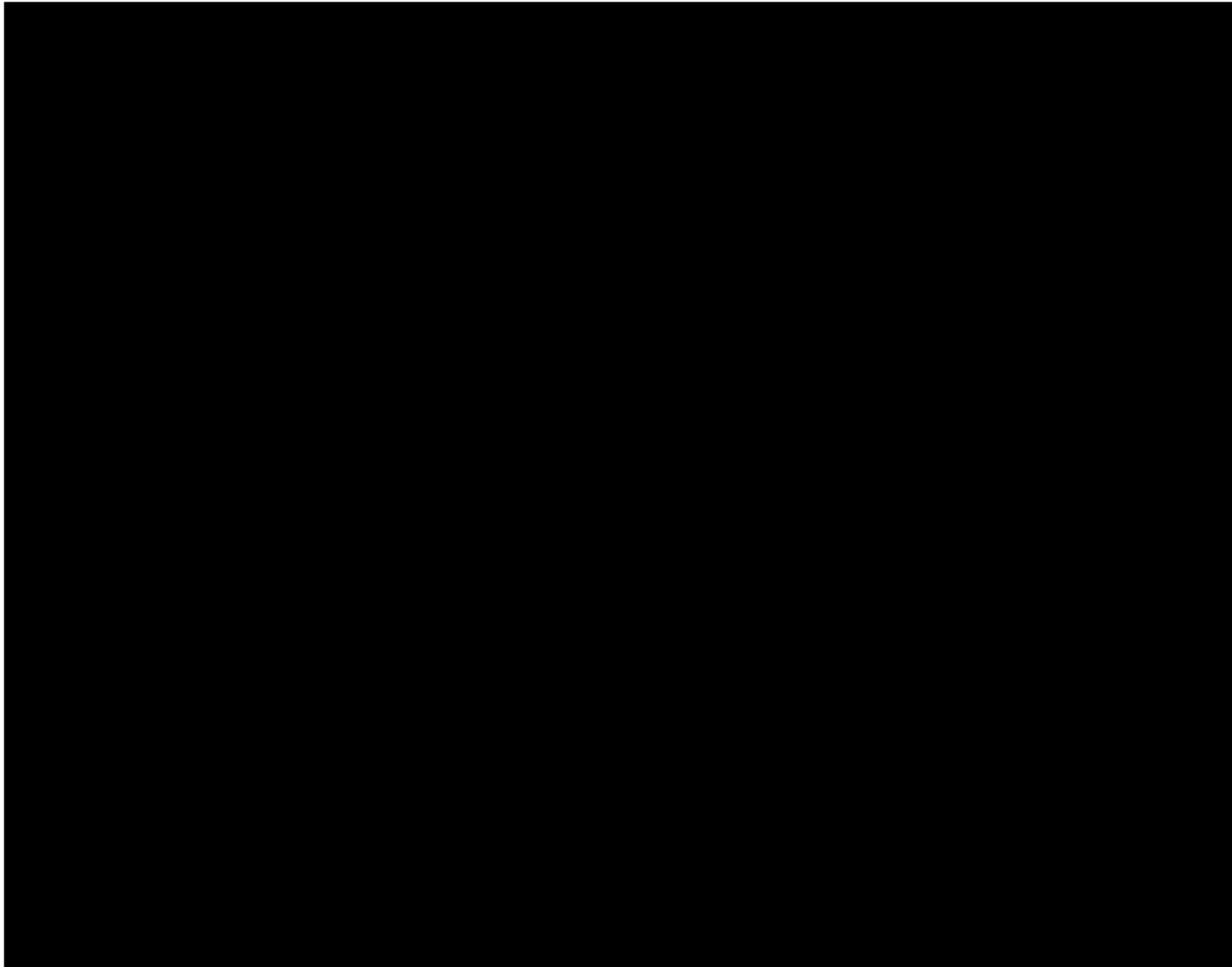


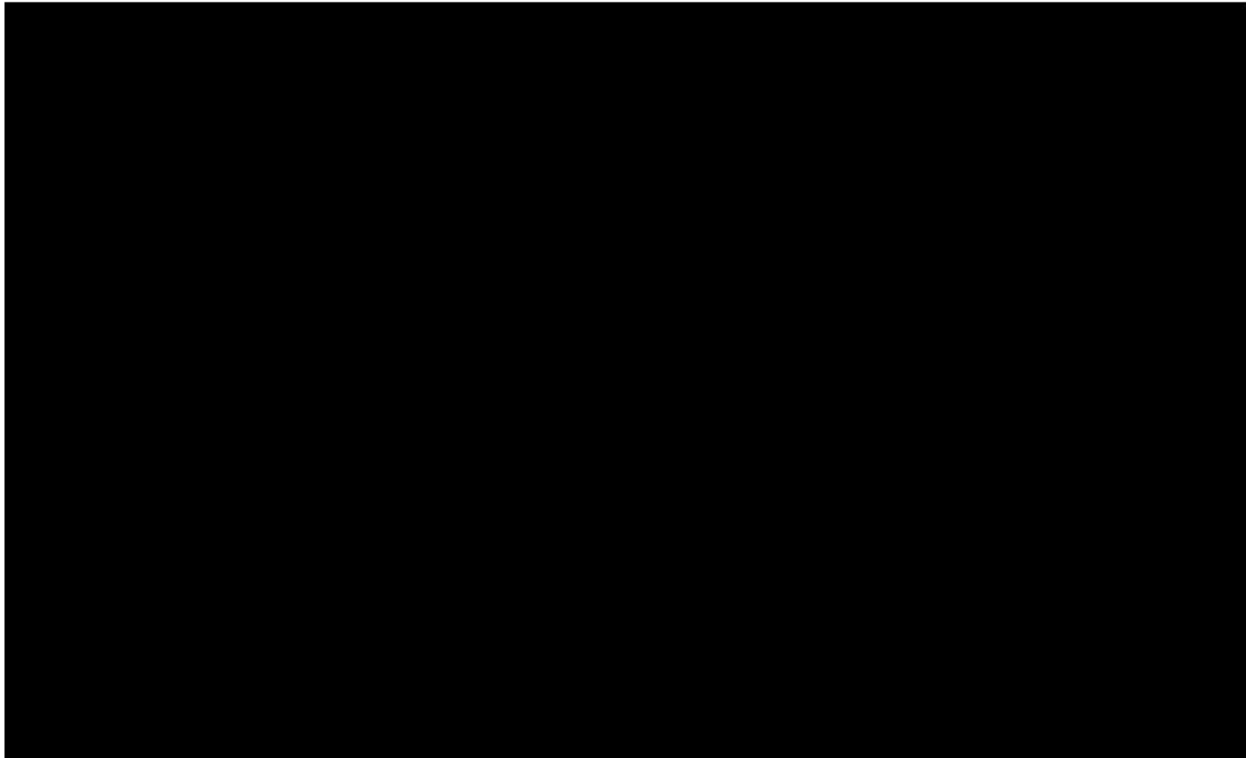
Figure 18. Map of Plasmid [illegible] for Southern Analysis
Schematic diagram of plasmid [illegible] indicating the [illegible] restriction enzyme sites with base pair position and the *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes between the [illegible] sites (indicated as Recombination Fragment Region in Figure 19) intended for insertion into the landing pad. The size of plasmid [illegible] is [illegible] bp



Number	Probe Name
1a & 1b	*pmi*
2	*mo-pat*
3	*ipd072Aa*

Figure 19. Map of the Recombination Fragment Region from Plasmid [redacted] for Southern Analysis

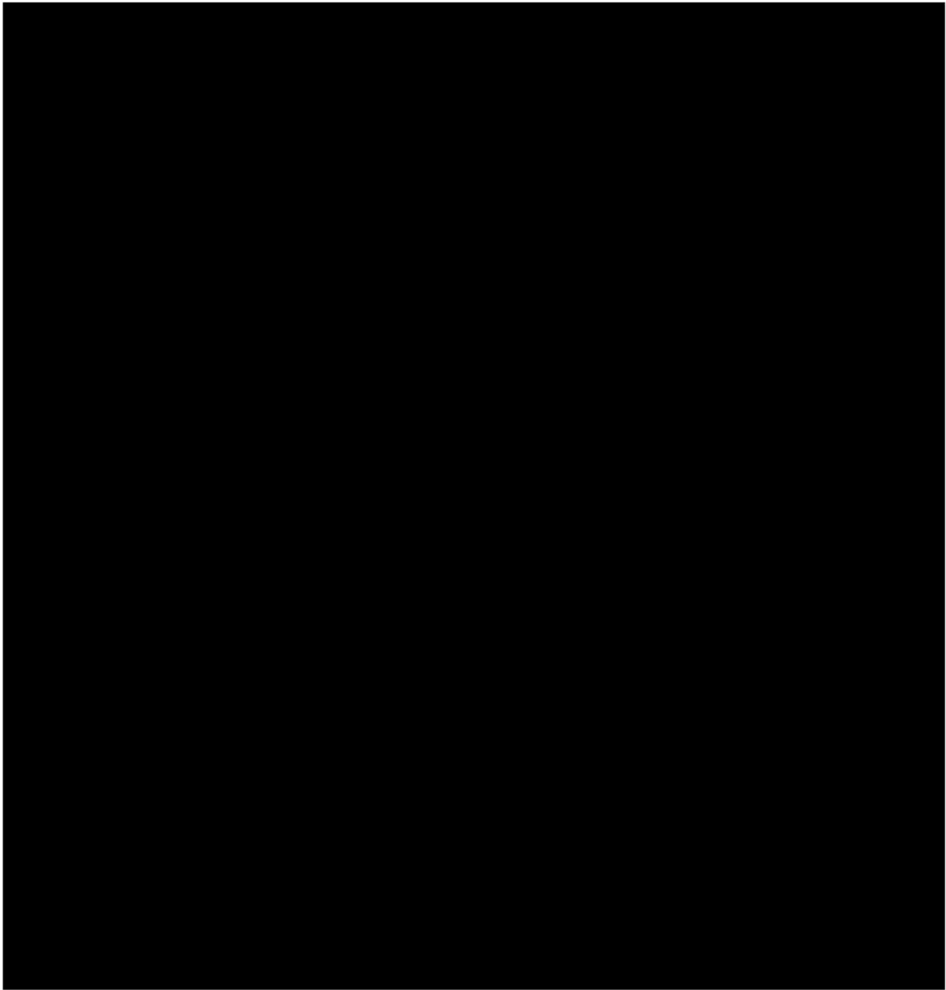
Schematic diagram of the [redacted] T-DNA indicating the [redacted] restriction enzyme site with base pair position and the *pmi, mo-pat*, and *ipd072Aa* gene cassettes between the [redacted] sites intended for insertion into the landing pad. The size of the [redacted] T-DNA is [redacted] bp. The genetic elements in the Recombination Fragment Region between the [redacted] sites were incorporated in the final DP51291 maize insertion (Figure 20). The locations of the Southern blot probes are shown by the boxes below the map.



Number	Probe Name
1a & 1b	*pmi*
2	*mo-pat*
3	*ipd072Aa*

Figure 20. Map of the DP51291 Insertion for Southern Analysis

Schematic diagram of the DP51291 maize insertion following SSI integration of the gene cassettes from the T-DNA (Figure 19) indicating the restriction enzyme sites. The DP51291 insert comprises sequences from two sources: the sequences of the landing pad outside the sites and the sequences from the T-DNA within the sites (with the *pmi*, *mo-pat*, and *ipd072Aa* gene cassettes). The flanking maize genomic regions are represented by the horizontal black rectangular bars. The locations of the Southern blot probes are shown by the boxes below the map. The 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.

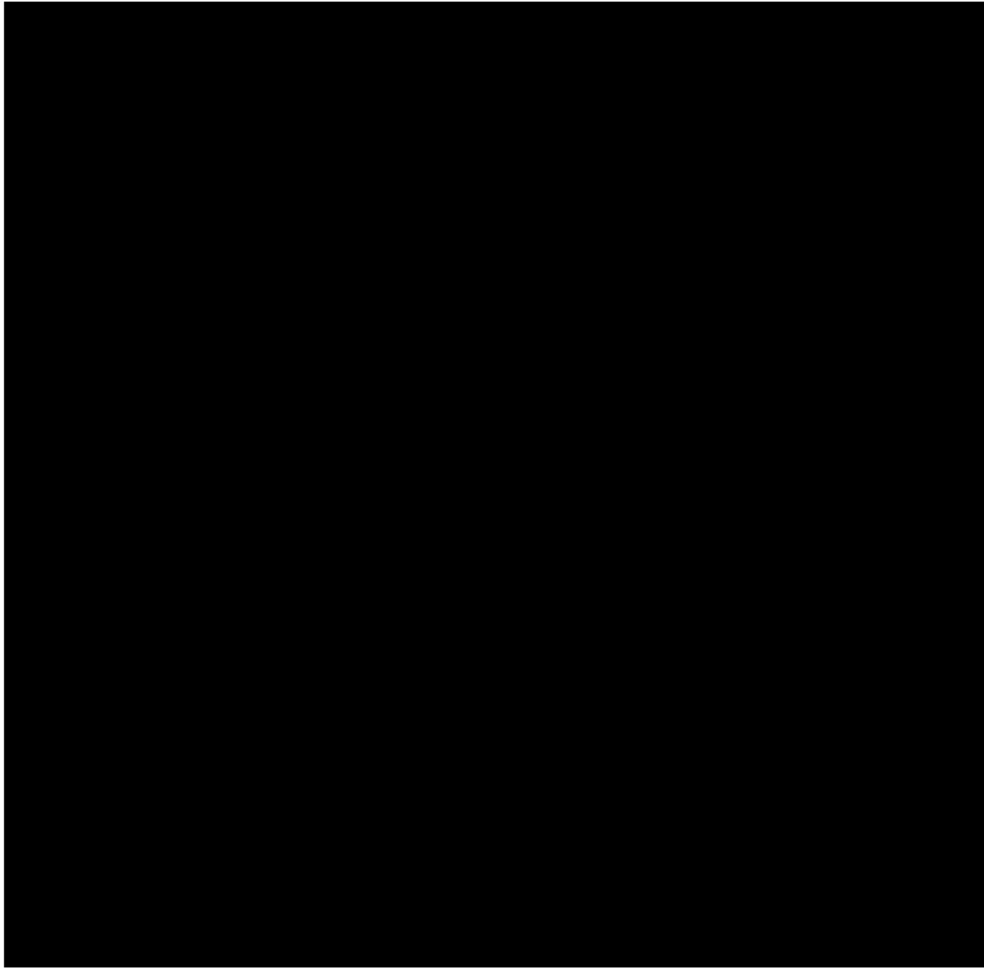


Lane	Sample
1	DIG-labeled DNA marker III
2	1 copy of [redacted] + PHR03 control maize
3	PHR03 control maize
4	Blank
5	DP51291 maize T1 generation
6	DP51291 maize T2 generation
7	DP51291 maize T3 generation

Lane	Sample
8	DP51291 maize T4 generation
9	DP51291 maize T5 generation
10	Blank
11	PHR03 control maize
12	1 copy of [redacted] + PHR03 control maize
13	DIG-labeled DNA marker VII

Figure 21. Southern Blot Analysis of of DP51291 Maize; [redacted] Digest with *pmi* Probe

Genomic DNA isolated from leaf tissues of DP51291 maize from T1, T2, T3, T4, and T5 generations and PHR03 control maize plants, was digested with [redacted] and hybridized to the *pmi* probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include [redacted] plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb)

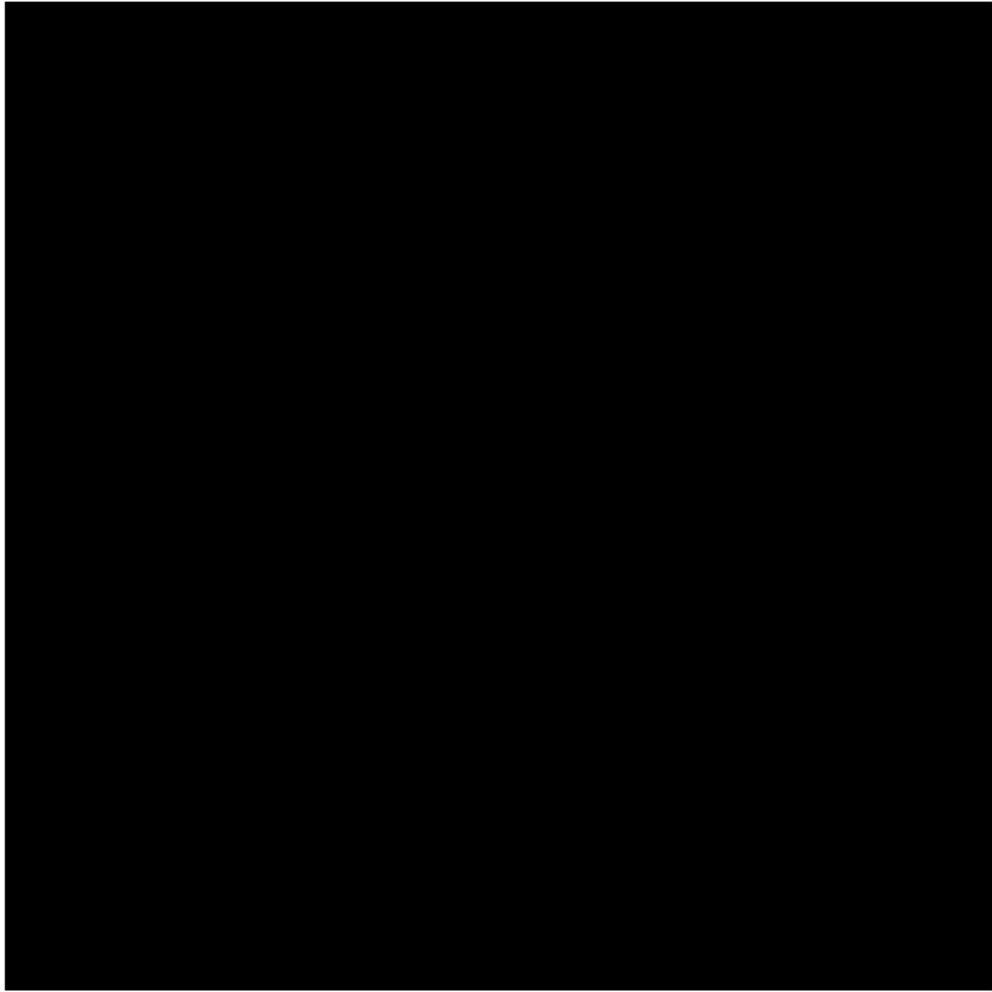


Lane	Sample
1	DIG-labeled DNA marker III
2	1 copy of [redacted] + PHR03 control maize
3	PHR03 control maize
4	Blank
5	DP51291 maize T1 generation
6	DP51291 maize T2 generation
7	DP51291 maize T3 generation

Lane	Sample
8	DP51291 maize T4 generation
9	DP51291 maize T5 generation
10	Blank
11	PHR03 control maize
12	1 copy of [redacted] + PHR03 control maize
13	DIG-labeled DNA marker VII

Figure 22. Southern Blot Analysis of DP51291 Maize; [redacted] Digest with *mo-pat* Probe

Genomic DNA isolated from leaf tissues of DP51291 maize from T1, T2, T3, T4, and T5 generations and PHR03 control maize plants, was digested with [redacted] and hybridized to the *mo-pat* probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include [redacted] plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).



Lane	Sample
1	DIG-labeled DNA marker III
2	1 copy of + PHR03 control maize
3	PHR03 control maize
4	Blank
5	DP51291 maize T1 generation
6	DP51291 maize T2 generation
7	DP51291 maize T3 generation

Lane	Sample
8	DP51291 maize T4 generation
9	DP51291 maize T5 generation
10	Blank
11	PHR03 control maize
12	1 copy of + PHR03 control maize
13	DIG-labeled DNA marker VII

Figure 23. Southern Blot Analysis of DP51291 Maize; Digest with *ipd072Aa* Probe

Genomic DNA isolated from leaf tissues of DP51291 maize from T1, T2, T3, T4, and T5 generations and PHR03 control maize plants, was digested with and hybridized to the *ipd072Aa* probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include plasmid DNA at approximately one gene copy number and 10 µg of control maize DNA. Sizes of the DIG-labeled DNA Molecular Weight Marker III and VII are indicated adjacent to the blot image in kilobases (kb).

Nucleotide Sequencing of the Introduced DNA and Genomic Flanking Regions

Sequence characterization analysis was performed to determine the DNA sequence of the DP51291 insert and flanking genomic regions.

Open Reading Frame Analysis of the Insert/Border Junctions ███ *2022 (PHI-2022-168/225 study))*

Assessing potentially expressed peptides (i.e., translated stop codon-bracketed frames) within an insertion or crossing the boundary between an insertion and its genomic borders for similarity to known and putative allergens and toxins is a critical part of the weight-of-evidence approach used to evaluate the safety of genetically modified plant products (Codex Alimentarius Commission, 2003). A bioinformatics assessment of translated stop codon-bracketed frames was conducted following established international criteria (Codex Alimentarius Commission, 2003; EFSA, 2010; EFSA, 2011; FAO/WHO, 2001). All translated stop codon-bracketed frames of length ≥ eight amino acids (aa) in the maize (*Zea mays* L.) event DP-Ø51291-2 (referred to as DP51291 maize) sequence that are within the insertion or that cross the boundary between the insertion and its genomic borders were identified and evaluated ███ 2019).

Seven hundred seventy-one (771) stop codon-bracketed frames ≥ eight aa were identified for the DP51291 maize sequence.

The allergen database used for the searches was the Comprehensive Protein Allergen Resource (COMPARE) 2022 database (January 2022), 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 2,463 sequences.

Searches of the translated stop codon-bracketed frames of DP51291 maize against the COMPARE allergen database revealed five (DP51291_235, DP51291_341, DP51291_562, DP51291_563, and DP51291_723) displaying > 35% identity with nine known allergens over "sliding windows" of 80 aa. The DP51291_235 translated stop codon-bracketed frame showed 35.6% to 37.0% identity (*E*-values from 0.03 to 7.1) to a bovine collagen α2 chain type 1 precursor (1364 aa; GenBank Accession NP_776945.1), 36.2% to 37.8% identity (*E*-values from 6.9 to 10) to a parasitic fish worm Ani s 11-like protein 2 precursor (287 aa; GenBank Accession BAJ78222.1), and 36.2% identity (*E*-values from 16 to 100) to rainbow trout collagen alpha 2 chain (1356 aa; GenBank Accession BAB55663.1). The DP51291_341 translated stop codon-bracketed frame showed 36.2% identity (*E*-values from 0.058 to 0.069) with a wheat partial high molecular weight glutenin (794 aa; GenBank Accession BAN29068), 36.2% identity (*E*-values from 0.059 to 0.070) to a wheat high molecular weight glutenin subunit (815 aa; GenBank Accession AAB02788.1), and 36.2% identity (*E*-values from 5.1 to 7.3) to another wheat high molecular weight glutenin subunit (830 aa; GenBank Accession CAA43331.1). The DP51291_562 translated stop codon-bracketed frame showed 36.2% identity (*E*-value = 0.00011) to a putative ragweed homolog of Art v 1 (164 aa; GenBank Accession CBJ24286.1) and 36.2% identity (*E*-value = 0.00011) to an allergen also described as a putative ragweed homolog of Art v 1 (164 aa; GenBank Accession CBK52317.1). The DP51291_563 translated stop codon-bracketed frame showed 35.4% identity (*E*-values from 0.046 to 0.065) to blue lupin conglutin beta 5 (637 aa; GenBank Accession F5B8W3.1) and 35.1% to 37.0% identity (*E*-values from 28 to 100) to rainbow trout collagen alpha 2 chain (1356 aa; GenBank Accession BAB55663.1). The DP51291_723 translated stop codon-bracketed frame showed 35.8% to 36.2% identity (*E*-values from 3.6 to 7.7) to rainbow trout collagen alpha 2 chain (1356 aa; GenBank Accession BAB55663.1).

While transcription of the region where the DP51291_235 frame is located would be expected given an upstream promoter element that drives the *mo-pat* gene (refer to Table 1 in ███ 2022 (PHI-2022-168/225 study)), the only

methionine residue (start codon) in DP51291_235 lies 19 amino acids from the C-terminal of the theoretical peptide, making only translation of a very short peptide viable. In any event, one would expect preferential translation of the intended PAT protein in this region. Furthermore, the methionine residue lies well downstream of the region of the frame involved in the alignments to allergens, so even if an unlikely translation product were produced, it would not be implicated in any possible cross-reactivity. In general, the alignments between translated frame DP51291_235 and the allergens are made possible only by gaps and the large number of proline, arginine, and glycine identities. Additionally, the alignments exhibit both low-level percent identities (> 35% to < 38%) and moderate to very high *E*-values (0.03 to 100).

Similarly, while transcription of the region where the DP51291_341 frame is located would be expected given an upstream promoter element that drives the *ipd072Aa* gene (refer to Table 1 in [redacted], 2022 (PHI-2022-168/225 study)), the only methionine residue in DP51291_341 lies precisely at the C-terminal of the theoretical peptide. In any event, one would expect preferential translation of the intended IPD072Aa protein in this region.

For DP51291_562, DP51291_563, and DP51291_723, there are no upstream promoter elements (refer to Table 3 in [redacted] 2022 (PHI-2022-168/225 study)), making transcription extremely unlikely. DP51291_562 and DP51291_563 lack methionine residues, making translation extremely unlikely even in the event of transcription. DP51291_723 does possess several methionine residues, but the fact that it almost certainly cannot be transcribed renders the presence of those moot. In any event, the alignments between translated frame DP51291_723 and the rainbow trout allergen are made possible only by gaps and the large number of proline and glycine identities. Additionally, the alignments exhibit both low-level percent identities (> 35% to < 37%) and high *E*-values (3.6 to 7.7).

Collectively, the results of this bioinformatics analysis suggest that the alignments produced between these five translated stop codon-bracketed frames and the allergens, when using an extremely conservative approach, are likely false positive hits without biological relevance and impart negligible risk of producing allergenic proteins. These data indicate that no allergenicity concerns arose from the alignments produced in the bioinformatics assessment of the translated stop codon-bracketed frames in DP51291 maize.

Four translated stop codon-bracketed frames (DP51291_110, DP51291_220, DP51291_409, and DP51291_562) produced 8-contiguous amino acid matches to allergens in the COMPARE allergen database. Upon analysis of the matches produced in this bioinformatics assessment of the translated stop codon-bracketed frames in DP51291 maize, no allergenicity concerns arose.

No alignments with an *E*-value ≤ 10^{-4} were returned between a translated stop codon-bracketed frame and any protein sequence in an internal toxin database. Twelve translated stop codon-bracketed frames produced alignments to proteins in the National Center for Biological Information (NCBI) non-redundant (nr) protein database with *E*-values ≤ 10^{-4}. None of the sequence alignments are related to any known toxic proteins that are harmful to humans or animals; while they do show expected alignments with non-toxic proteins, many are already present in the food and/or feed chain. Therefore, no toxicity concerns arose from the bioinformatics assessment of the translated stop codon-bracketed frames in DP51291 maize.

Bioinformatics evaluation of the DP51291 insert did not generate biologically relevant amino acid sequence similarities to known allergens, toxins, or other proteins that would be harmful to humans or animals.

Event-Specific Detection Methodology

The event-specific quantitative real-time PCR method for DP51291 maize was developed and validated to measure the relative content of DP51291 maize to total maize DNA utilizing standard curves for both the taxon-specific High Mobility Group (HMG) and DP51291 maize assays ████ 2022 (PHI-2022-092 study)). The relative content of the DP51291 maize was determined using the ratio between the mean copy number of the DP51291 maize insertion event compared to the haploid maize genome. The testing results of the event-specific quantitative real-time PCR method for DP51291 maize demonstrates that this method fulfils the internationally accepted minimum performance requirements for analytical methods of GMO testing.

Conclusions on the Molecular Characterisation of DP51291 Maize

SbS, Southern blot, multi-generation segregation, Sanger sequencing of the insert and genomic border regions and bioinformatic analysis of the genomic border regions and putative translated ORFs, were conducted to characterize the inserted DNA in DP51291 maize.

SbS analysis confirmed that DP51291 maize contains a single copy of the inserted DNA with the expected organization, and that no additional insertions, plasmid backbone, or other unintended plasmid sequences are present in DP51291 maize. Southern blot analysis of five generations of DP51291 maize confirmed that the inserted DNA in DP51291 maize is consistent and stable across multiple generations during the breeding process.

Segregation analysis (refer to Section A.3(e) *Stability of genetic changes*) confirmed that the inserted DNA in DP51291 maize segregated as a single locus according to Mendelian rules of inheritance across five generations, and the stability of the insertion and of the herbicide tolerance phenotype was demonstrated in these populations. A bioinformatics evaluation of the DP51291 maize insert did not generate biologically relevant amino acid sequence similarities to known allergens, toxins, or other proteins that would be harmful to humans or animals.

Sanger sequencing analyses determined the sequence of the insert and flanking genomic regions in DP51291 maize. The total length of sequence determined in DP51291 maize is █████ base pairs (bp), comprised of ████ bp of the 5' flanking genomic sequence; ████ bp of the 3' flanking genomic sequence; and █████ bp of the inserted DNA. Upon comparing the sequence of the DP51291 insert with the landing pad from ███████ T-DNA and the recombination fragment region from ██████, the DP51291 insert was confirmed to have the expected sequence from ███████ and ███████ except █ bp from the ████████ and █ bp from the ███████ were incorporated into the genome. All remaining sequence is intact and identical to the sequences derived from plasmids ███████ and ███████ In addition, the Sanger sequencing result confirms the three gene cassettes (*ipd072Aa*, *mo-pat*, and *pmi*) of the DP51291 insert are identical to the corresponding components of the insertion in previously assessed and authorized corn line DP23211.

Together, these analyses confirmed that a single copy of the inserted DNA, with no plasmid backbone sequences or other unintended plasmid sequences, is present in the DP51291 maize genome. The introduced genes are stably inherited across multiple generations and segregated according to Mendel's law of inheritance during the breeding process. Sanger sequencing analyses determined the sequences of the inserted DNA and flanking genomic regions in DP51291 maize. Bioinformatic analyses support the conclusion that there is unlikely to be allergenicity or toxicity concerns regarding the putative translated ORFs at the DP51291 insertion site. Additionally, an event-specific quantitative real-time PCR detection method was developed and validated for DP51291 maize.

A.3(d) Selection and breeding process

Selection of Maize Event DP51291

Plants that were regenerated from transformation and tissue culture (designated T0 plants) were selected for further characterization. A schematic overview of the transformation and event development process for DP51291 maize is provided in Figure 24.

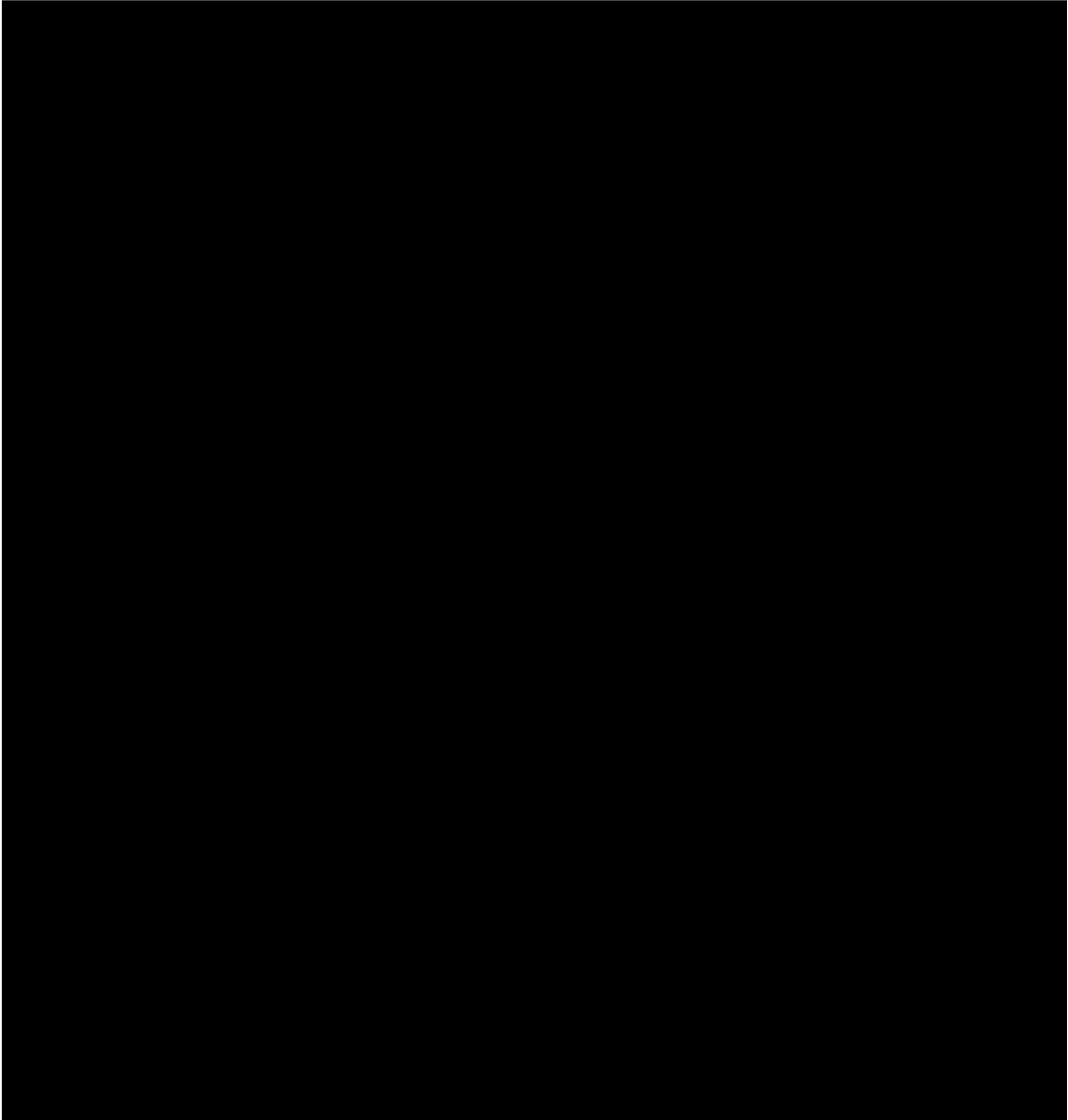


Figure 24. Event Development Process of DP51291 Maize

Breeding Method

The subsequent breeding of DP51291 maize proceeded as indicated in Figure 25 to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial maize lines. Table 10 provides the generations used for each characterization study.

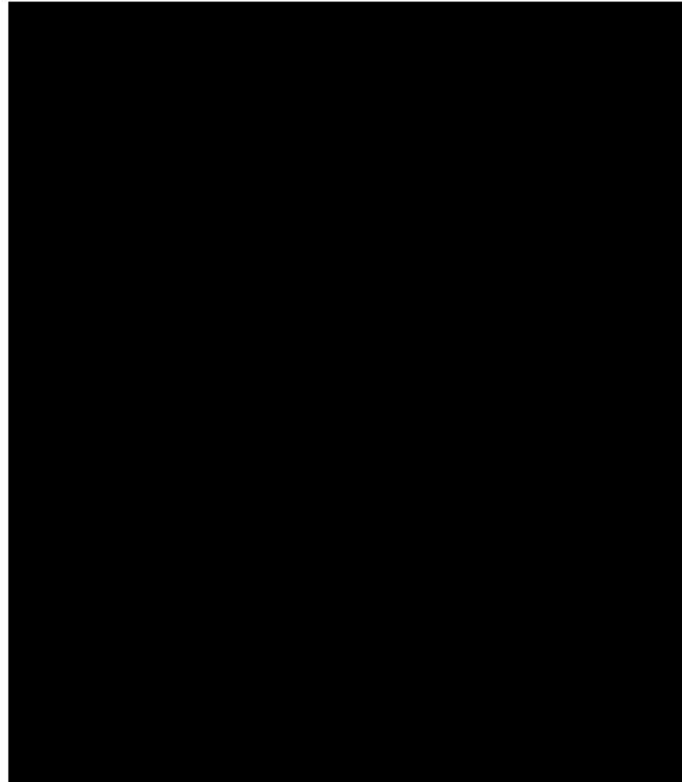


Figure 25. Breeding Diagram for DP51291 Maize and Generations Used for Analysis

The breeding steps to produce the generations used for characterization, assessment, and the development of commercial lines are shown schematically. Proprietary maize inbred PHR03 was used for transformation to produce DP51291 maize.

Table 10. Generations and Comparators Used for Analysis of DP51291 Maize

Analysis	Seed Generation(s) Used	Comparators
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

A.3(e) Stability of genetic changes

Multi-Generation Segregation Analysis [redacted] 2022 (PHI-2018-035 study))

Segregation analysis was performed on five generations of DP51291 maize (T1, T2, T3, T4, and T5 generations) to confirm the Mendelian inheritance pattern of the inserted DNA during the breeding process. The observed inheritance pattern predicts the segregation of these genes and/or traits as a single unit and as a single genetic locus throughout the commercial breeding process. A total of 100 maize plants from each generation of DP51291 maize were analyzed using genotypic and phenotypic analyses.

The genotypic analyses utilized quantitative real-time polymerase chain reaction (qPCR) analysis to determine the copy number of the [redacted] insertion site representing event DP-Ø51291-2 and the *ipd072Aa, mo-pat,* and *pmi* genes as well as qualitative PCR analysis (PCR) to determine the presence or absence of specific genetic elements. The phenotypic analysis utilized a visual herbicide injury evaluation to confirm the presence or absence of tolerance to glufosinate for each individual plant. The individual results for each plant were compared to the genotypic qPCR and PCR results to verify co-segregation of genotype and phenotype. A chi-square test was performed at the 0.05 significance level to compare the observed segregation ratios of T1, T2, T3, T4 and T5 generations of DP51291 maize to the expected segregation ratios (1 :1 for T1 generation, 3:1 for T2 and T3 generations). A chi-square test was not performed for the T4 and T5 generations of DP51291 maize as all plants were identified as positive as expected for a homozygous generation.

A summary of segregation results for DP51291 maize (T1, T2, T3, T4, and T5 generations) is provided in Table 11. No statistically significant deviation from the expected segregation ratio was found in the T1, T2, and T3 generations of DP51291 maize, and the T4 and T5 generations were confirmed to be non-segregating. Genotypic results based on the genotypic qPCR and PCR results demonstrated that the observed segregation ratios matched the expected segregation ratios for all five generations. Results of phenotypic analysis for tolerance to glufosinate herbicide aligned with the genotypic results.

The results of the multi-generation segregation analysis demonstrated that the inserted DNA in DP51291 maize segregated as a single locus in accordance with Mendelian rules of inheritance for a single genetic locus, indicating stable integration of the insert into the maize genome and a stable genetic inheritance pattern across generations.

Additional details regarding analytical methods for the multi-generation segregation analysis are provided in Appendix C.

Table 11. Summary of Genotypic and Phenotypic Segregation Results for Five Generations of DP51291 Maize

Generation	Expected Segregation Ratio	Observed Segregation			Statistical Analysis	
	(Positive:Negative)	Positive	Negative	Total	Chi-Square[a]	P-Value
T1	1:1	49	51	100	0.04	0.8415
T2	3:1	82	18	100	2.61	0.1060
T3	3:1	74	26	100	0.05	0.8174
T4	Homo	100	0	100	--	--
T5	Homo	100	0	100	--	--

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

Southern Analysis to Determine Stable Genetic Inheritance [redacted] 2022 (PHI-2022-064 study))

For the multi-generation Southern analysis to determine stable generic inheritance, see Section *A.3(c) Molecular characterisation*.

B CHARACTERISATION AND SAFETY ASSESSMENT OF THE NEW SUBSTANCES

B.1 CHARACTERISATION AND SAFETY ASSESSMENT OF NEW SUBSTANCES

There are no new substances associated with DP51291 maize other than the three proteins (see Section B.2 below).

B.2 NEW PROTEINS

IPD072Aa protein

Amino Acid Sequence

The deduced amino acid sequence from the translation of the *ipd072Aa* gene in DP51291 maize is 86 amino acids in length and has a molecular weight of approximately 10 kDa. The protein characterization results via SDS-PAGE, western blot, peptide mapping, N-terminal amino acid sequence demonstrated that the IPD072Aa protein derived from DP51291 maize is of the expected molecular weight, immunoreactivity, amino acid sequence and showed a lack of glycosylation (see section below). The IPD072 protein expressed in DP51291 is identical to that of the IPD072 protein that is found in the authorized maize event DP-Ø23211-2 (DP23211 maize). Therefore, the previously conducted safety studies to assess IPD072 protein for DP23211 maize would also be relevant and applicable to IPD072 protein in DP51291 maize.

```
IPD072Aa (DP51291)  1 MGITVTNNSS NPIEVAINHW GSDGDTSFFS VGNGKQETWD RSDSRGFVLS
IPD072Aa (DP23211)  1 MGITVTNNSS NPIEVAINHW GSDGDTSFFS VGNGKQETWD RSDSRGFVLS

IPD072Aa (DP51291) 51 LKKNGAQHPY YVQASSKIEV DNNAVKDQGR LIEPLS*
IPD072Aa (DP23211) 51 LKKNGAQHPY YVQASSKIEV DNNAVKDQGR LIEPLS*
```

Figure 26. Alignment of the Deduced Amino Acid Sequence of the IPD072Aa Protein
Deduced amino acid sequence alignment, where IPD072Aa (DP51291) represents the deduced amino acid sequence from the translation of the *ipd072Aa* gene from plasmid [redacted] used to generate DP51291 maize. The IPD072Aa (DP23211) that is found in the authorized corn line DP23211 (Application A1202). The asterisk (*) indicates the translational stop codon. The full-length protein is 86 amino acids in length and has a molecular weight of approximately 10 kDa.

Function and Activity of the IPD072Aa Protein

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

Characterization of the IPD072Aa Protein Derived from DP51291 Maize

The IPD072Aa protein was purified from DP51291 maize whole plant tissue using ammonium sulfate precipitation and immuno-affinity chromatography.

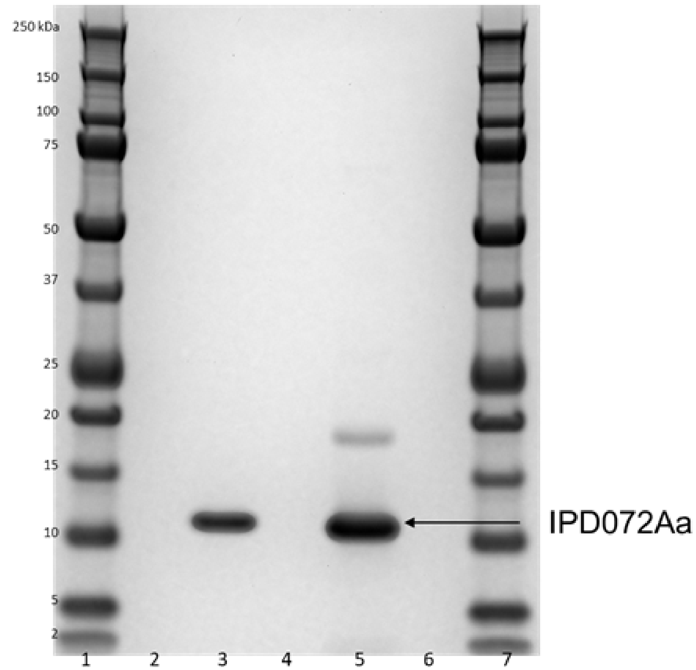
In order to have sufficient amounts of purified IPD072Aa protein for the multiple studies required to assess its safety, IPD072Aa protein was expressed in an *Escherichia coli* protein expression system as a fusion protein with an N-terminal histidine tag.

The biochemical characteristics of the DP51291 maize-derived IPD072Aa protein were characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, glycosylation, mass spectrometry, and N-terminal amino acid sequence analyses. The results showed that DP51291 maize-derived IPD072Aa protein is of the expected molecular weight, immunoreactivity, lack of glysoylation, and amino acid sequence. The microbially derived IPD072Aa protein used as a reference is the same test reference that was used in safety studies for recently reviewed and authorized corn line DP23211 (Application A1202).

SDS-PAGE Analysis

Samples of the DP51291 maize-derived IPD072Aa protein and the microbially derived IPD072Aa protein were analysed by SDS-PAGE (2022 (PHI-2022-054 study)). As expected, the IPD072Aa proteins derived from both DP51291 maize and the microbial system migrated as a predominant band consistent with the expected molecular weight of approximately 10 kDa (Carlson et al., 2019), as shown in Figure 27.

Additional details regarding SDS-PAGE analytical methods are provided in Appendix D.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	Microbially Derived IPD072Aa Protein (Lot # PCF-0037-AP)[b]
4	1X LDS/DTT Sample Buffer Blank
5	DP51291 Maize-Derived IPD072Aa Protein
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

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

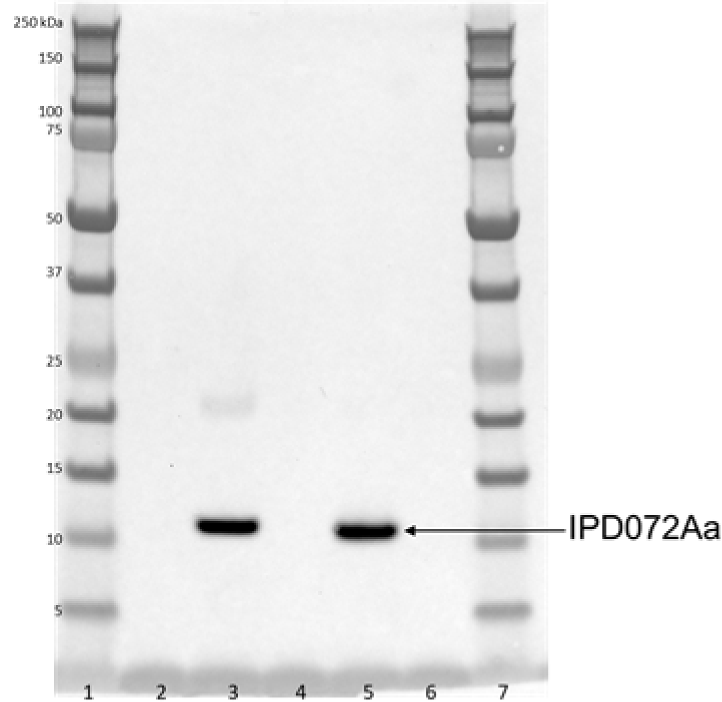
[b] Target loading amount of 1 µg.

Figure 27. SDS-PAGE Analysis of the IPD072Aa Protein

Western Blot Analysis

Samples of the DP51291 maize-derived IPD072Aa protein and the microbially derived IPD072Aa protein were analyzed by western blot (█████████ 2022 (PHI-2022-054 study)). As expected, the IPD072Aa proteins derived from both DP51291 maize and the microbial system were immunoreactive to a IPD072Aa polyclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 10 kDa(Carlson *et al.*, 2019), as shown in Figure 28.

Additional details regarding western blot analytical methods are provided in Appendix D.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	Microbially Derived IPD072Aa Protein (Lot # PCF-0037-AP)[b]
4	1X LDS/DTT Sample Buffer Blank
5	DP51291 Maize-Derived IPD072Aa Protein
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

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

[b] Target loading amount of 10 ng.

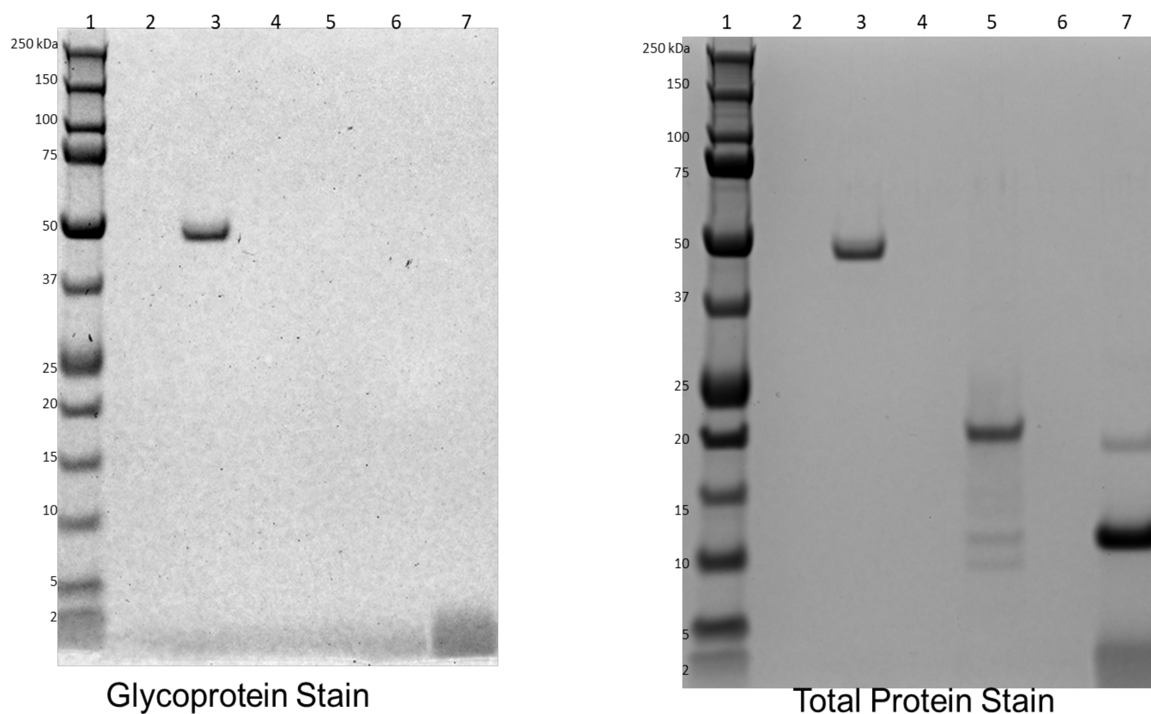
Figure 28. Western Blot Analysis of the IPD072Aa Protein

Glycoprotein Analysis

Samples of the DP51291 maize-derived IPD072Aa protein was analyzed by SDS-PAGE for glycosylation analysis ([redacted] 2022(PHI-2022-054 study)). Each gel also included a positive control (horseradish peroxidase) and a negative control (soybean trypsin inhibitor). The gels were then stained using a Pierce Glycoprotein Staining Kit to visualize any glycoproteins. The gels were imaged and then stained with GelCode Blue stain reagent to visualize all protein bands.

Glycosylation was determined to be negative for the DP51291 maize-derived protein (Figure 29). The horseradish peroxidase positive control was clearly visible as a stained band. The soybean trypsin inhibitor negative control was not stained by the glycoprotein stain.

Additional details regarding glycoprotein analytical methods are provided in Appendix D.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	Horseradish Peroxidase (1.0 µg)
4	1X LDS/DTT Sample Buffer Blank
5	Soybean Trypsin Inhibitor (1.0 µg)
6	1X LDS/DTT Sample Buffer Blank
7	DP51291 Maize-Derived IPD072Aa Protein

Note: The glycoprotein gel was stained with glycoprotein staining reagent. The total protein stain gel was stained with glycoprotein staining reagent followed by staining with Coomassie blue reagent for total protein. Kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

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

Figure 29. Glycosylation Analysis of DP51291 Maize-Derived IPD072Aa Protein

Mass Spectrometry Peptide Mapping Analysis

Samples of the DP51291 maize-derived IPD072Aa protein were analyzed by SDS-PAGE. Protein bands were stained with Coomassie stain reagent, and the band containing IPD072Aa protein was excised for each sample.

The excised IPD072Aa protein bands derived from DP51291 maize were digested with trypsin and chymotrypsin. Digested samples were analyzed using liquid chromatography-mass spectrometry (LC-MS) [redacted] 2022 (PHI-2022-054 study)). The resulting MS data were used to search and match the peptides from the expected IPD072Aa protein sequence. The identified tryptic and chymotrypic peptides for the DP51291 maize-derived IPD072Aa protein are shown in Table 12. The combined sequence coverage of the identified tryptic and chymotrypic peptides for DP51291 maize-derived IPD072Aa protein account for 61.2% (52/85) of the expected amino acid sequence (Table 13 and Figure 30).

Additional details regarding peptide mapping analytical methods are provided in Appendix D.

Table 12. Identified Tryptic Peptides of DP51291 Maize-Derived IPD072Aa Protein Using LC MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
Tryptic Peptides			
45-52	890.5602	890.5589	GFVLSLKK
52-66	1676.8224	1676.8270	KNGAQHPYYVQASSK
53-66	1548.7237	1548.7321	NGAQHPYYVQASSK
67-75	1000.4465	1000.5189	IEVDNNAVK
67-79	1456.6325	1456.7270	IEVDNNAVKDQGR
Chymotryptic Peptides			
28-38	1251.5883	1251.5884	FSVGNGKQETW
29-38	1104.5188	1104.5200	SVGNGKQETW
39-46	938.4176	938.4206	DRSDSRGF
39-48	1150.5773	1150.5731	DRSDSRGFVL

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

Table 13. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived IPD072Aa Protein Using LC-MS Analysis

Protease	% Coverage	Combined % Coverage
Trypsin	41	61.2
Chymotrypsin	24	

1 GITVTNNSSN PIEVAINHWG SDGDTSF**FSV** **GNGKQETWDR** **SDSRGFVLSL**
51 **KKNGAQHPYY** **VQASSKIEVD** **NNAVKDQGR**L IEPLS

Red bold type	Red bold type indicates DP51291 maize-derived IPD072Aa peptides identified using LC-MS analysis against the expected IPD072Aa protein sequence.
Amino acid residue abbreviations	alanine (A), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V)

Note: The expected IPD072Aa protein sequence does not include the N-terminal methionine as it is anticipated to be absent.

Figure 30. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DP51291 Maize-Derived IPD072Aa Protein Using LC-MS Analysis

N-Terminal Amino Acid Sequence Analysis

The Edman sequencing analysis of the DP51291 maize-derived IPD072Aa protein sample determined the N-terminal sequence (GITVTNNSSN) matching amino acid residues 1-10 of the expected protein sequence (Table 14), indicating the N terminal methionine was absent as anticipated (Dummitt *et al.*, 2003; Sherman *et al.*, 1985). The determined N-terminal sequence of DP51291 maize-dervied IPD072Aa protein is identical to the determined N-terminal sequence of the IPD072Aa protein derived from previously assessed and authorized maize event DP-Ø23211-2. Additionally, the analysis of the microbially derived IPD072Aa protein using Edman sequencing identified an N-terminal sequence (HMGITVTNNS), matching amino acid residues 1-10 of the expected sequence (Carlson *et al.*, 2019) (Table 14).

Additional details regarding N-terminal amino acid sequencing analytical methods are provided in Appendix D.

Table 14. N-Terminal Amino Acid Sequence Analysis of the IPD072Aa Protein

Description		Amino Acid Sequence
Maize-Derived IPD072Aa Protein	Observed Sequence (DP51291 Maize)	G – I – T – V – T – N – N – S – S – N
	Observed Sequence (DP23211 Maize)	G – I – T – V – T – N – N – S – S – N
Microbially Derived IPD072Aa Protein	Expected Sequence	H – M – G – I – T – V – T – N – N – S
	Observed Sequence (Tox Lot PCF-0037-AP)	H – M – G – I – T – V – T – N – N – S
	Observed Sequence (Tox Lot PCF-0040)	H – M – G – I – T – V – T – N – N – S

Note: The N-terminal methionine in the detected primary sequence for the IDP072Aa protein derived from DP51291 maize and DP23211 maize was absent as expected. Asparagine (N), glycine (G), histidine (H), isoleucine (I), methionine (M), serine (S), threonine (T), and valine (V).

Allergenicity and Toxicity Analyses of the IPD072Aa Protein

The IPD072Aa protein encoded by the *ipd072Aa* gene in DP51291 maize is identical to the IPD072Aa protein encoded by the *ipd072Aa* gene that is found in the authorized herbicide-tolerant and insect-protected corn line DP23211 listed in Schedule 26 *Food produced using gene technology,* entry (zd) for corn. The IPD072Aa protein safety data has been previously reviewed by FSANZ (see A1202 application). In accordance with the FSANZ Application Handbook, only the updated bioinformatics analysis is provided in this dossier for safety assessment of the IPD072Aa protein.

Bioinformatic Analysis of Homology to Known or Putative Allergens and Toxins ██████, 2022 (PHI-2022-168/225 study))

A bioinformatics assessment of translated stop codon-bracketed frames was conducted according to relevant guidelines (Codex Alimentarius Commission, 2009; FAO/WHO, 2001). All translated stop codon-bracketed frames of length ≥ eight amino acids in the maize (*Zea mays* L.) event DP-Ø51291-2 (referred to as DP51291 maize) sequence that are within the insertion or that cross the boundary between the insertion and its genomic borders were identified and evaluated. A full description is located in ██████ 2022 (PHI-2022-168/225 study). This report includes the bioinformatic analysis for the IPD072Aa protein coding sequence (DP51291_340). For information on the IPD072Aa protein coding sequence alignments, please refer to Table 3, specifically Frame DP51291_340 in ██████ 2022 (PHI-2022-168/225 study). In conclusion, bioinformatics evaluation of the DP51291 insert did not generate

biologically relevant amino acid sequence similarities to known allergens, toxins, or other proteins that would be harmful to humans or animals.

Conclusions on the Safety of IPD072Aa Protein in DP51291 Maize

The protein characterization results via SDS-PAGE, western blot, peptide mapping, N-terminal amino acid sequence demonstrated that the IPD072Aa protein derived from DP51291 maize is of the expected molecular weight, immunoreactivity, amino acid sequence and showed a lack of glycosylation. The amino acid sequence of the IDP072Aa protein present in DP51291 maize was demonstrated to be identical to the IDP072Aa protein previously evaluated for the herbicide-tolerant and insect-protected corn line DP23211 and concluded to be safe (A1202 application). Updated bioinformatics comparisons of the IPD072Aa protein sequence to known or putative allergen and toxin sequences support the original conclusions that the IPD072Aa protein is unlikely to be allergenic or toxic to humans or animals. Based on this weight of evidence, consumption of the IPD072Aa protein is unlikely to cause an adverse effect on humans or animals.

PAT protein

Amino Acid Sequence

The gene encoding the PAT protein in DP51291 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 33; [redacted] 2018).

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

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

As shown in Figure 31, 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 (Hérouet et al., 2005) in a number of approved events across several different crops that are currently in commercial use.

Function and Activity of the PAT Protein

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

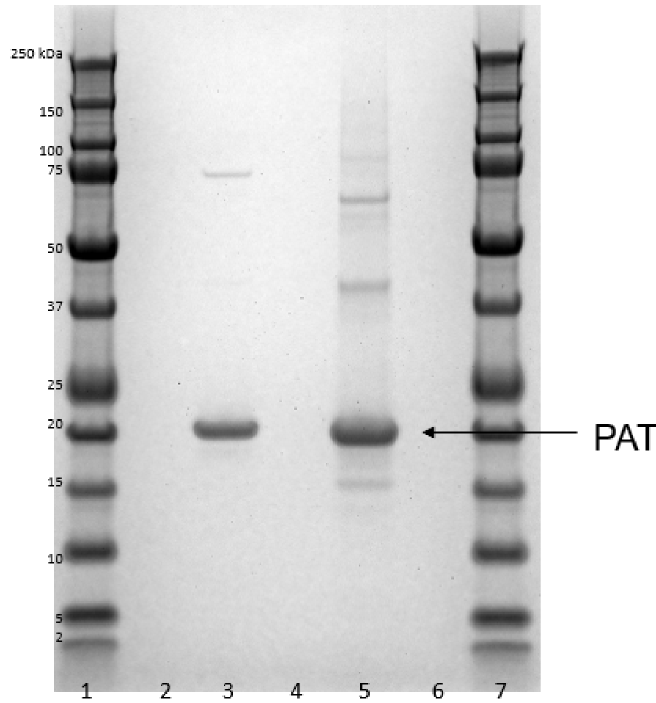
Characterisation of the PAT Protein Derived from DP51291 Maize

The DP51291 maize-expressed PAT protein was characterized using SDS-PAGE analysis, western blot analysis, protein glycosylation analysis, mass spectrometry peptide mapping, and N-terminal amino acid sequence analyses. The results demonstrated that the PAT protein derived from DP51291 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation ([redacted] 2022 (PHI-2022-055 study)).

SDS-PAGE Analysis

Samples of PAT protein purified from DP51291 maize whole plant tissue were analyzed by SDS-PAGE. As expected, all PAT protein samples migrated as a predominant band consistent with the expected molecular weight of approximately 21 kDa (Figure 32).

Additional details regarding SDS-PAGE analytical methods are provided in Appendix E.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	PAT Protein Reference Substance (Lot # PCF-0038; 1 µg)
4	1X LDS/DTT Sample Buffer Blank
5	DP51291 Maize-Derived PAT Protein (1:6 dilution)
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

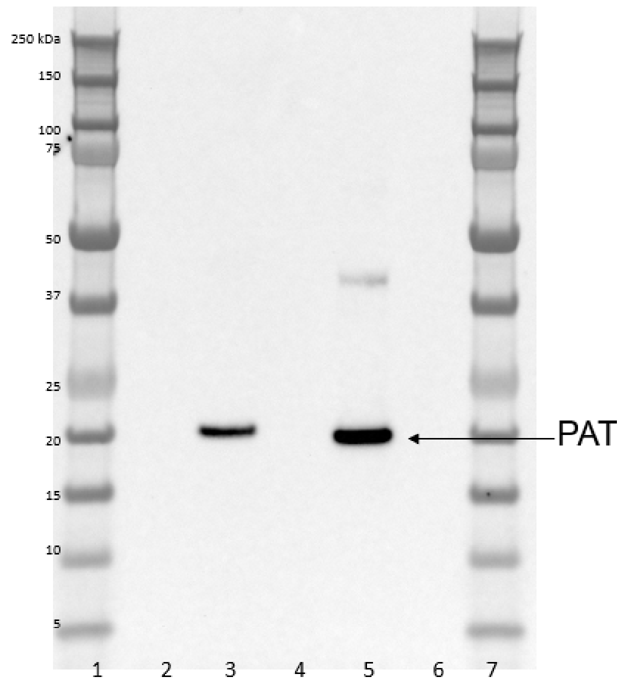
[a] Molecular weight markers were included to provide a visual verification that migration was within the expected range of the predicted molecular weight.

Figure 32. SDS-PAGE Analysis of DP51291 Maize-Derived PAT Protein

Western Blot Analysis

Samples of PAT protein purified from DP51291 maize whole plant tissue were analyzed by western blot. As expected, all PAT protein samples were immunoreactive to a PAT monoclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 21 kDa (Figure 33).

Additional details regarding western blot analytical methods are provided in Appendix E.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	PAT Protein Reference Substance (Lot # PCF-0038; 10 ng)
4	1X LDS/DTT Sample Buffer Blank
5	DP51291 Maize-Derived PAT Protein (1:600 dilution)
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).
[a] Molecular weight markers were included to provide a visual verification that migration was within the expected range of the predicted molecular weight.

Figure 33. Western Blot Analysis of DP51291 Maize-Derived PAT

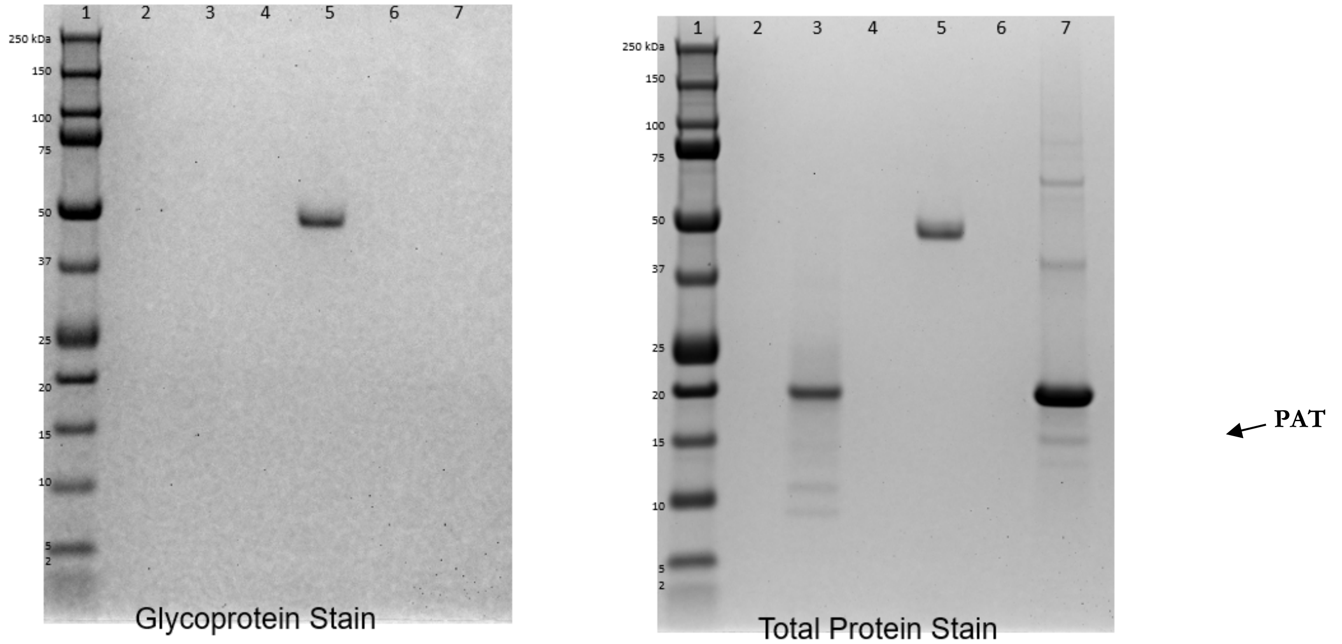
Glycosylation Analysis

Samples of PAT protein purified from DP51291 maize whole plant tissue were analyzed by SDS-PAGE. Each gel also included a positive control (horseradish peroxidase) and a negative control (soybean trypsin inhibitor). The gels were stained using a Pierce Glycoprotein Staining Kit to visualize any glycoproteins. The gels were imaged and then stained with GelCode Blue stain reagent to visualize all protein bands.

Glycosylation was not detected for the PAT protein (Figure 34). The horseradish peroxidase positive control was

stained and clearly visible as a magenta-colored band. The soybean trypsin inhibitor negative control was not stained by the glycoprotein stain.

Additional details regarding glycosylation analytical methods are provided in Appendix E.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	Soybean Trypsin Inhibitor (1.0 µg)
4	1X LDS/DTT Sample Buffer Blank
5	Horseradish Peroxidase (1.0 µg)
6	1X LDS/DTT Sample Buffer Blank
7	DP51291 Maize-Derived PAT Protein (1:6 dilution)

Note: The glycoprotein gel was stained with glycoprotein staining reagent. The total protein stain gel was stained with glycoprotein staining reagent followed by staining with Coomassie blue reagent for total proteins. Kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

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

Figure 34. Glycosylation Analysis of DP51291 Maize-Derived PAT Protein

LC-MS Peptide Mapping Analysis

Samples of the PAT protein purified from DP51291 maize whole plant tissue were analyzed by SDS-PAGE. Protein bands were stained with Coomassie stain reagent, and the band containing PAT protein was excised for each sample. The excised PAT protein bands were digested with trypsin or chymotrypsin. Digested samples were analyzed using LC-MS, and an MS/MS ion search was used to match the detected peaks to peptides from the expected PAT protein sequence.

The identified tryptic and chymotrypic peptides for DP51291 maize-derived PAT protein are shown in Table 15. The combined sequence coverage was 95.6% (174/182) of the expected PAT amino acid sequence (Table 16). The deduced amino acid sequence includes an additional amino acid, an N-terminal methionine, that is not included in the expected 182-amino acid sequence for DP51291 maize used in LC-MS analysis (Figure 35).

Additional details regarding peptide mapping analytical methods are provided in Appendix E.

Table 15. Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived PAT Protein Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
Tryptic Peptides			
5–36	3615.8148	3615.7926	RPVEIRPATAADMAAVCDIVNHYIETSTVNFR
37–51	1855.8677	1855.8588	TEPQTPQEWIDDLER
52–77	2886.5259	2886.5068	LQDRYPWLVAEVEGVVAGIAYAGPWK
56–77	2374.2736	2374.2361	YPWLVAEVEGVVAGIAYAGPWK
78–95	2153.0270	2153.0290	ARNAYDWTVESTVYVSHR
80–95	1925.8068	1925.8908	NAYDWTVESTVYVSHR
99–111	1414.8241	1414.8184	LGLGSTLYTHLLK
112–119	896.3412	896.4062	SMEAQGFK
112–134	2400.2669	2400.2471	SMEAQGFKSVVAVIGLPNDPSVR
120–134	1521.8594	1521.8515	SVVAVIGLPNDPSVR
135–144	1129.4926	1129.5880	LHEALGYTAR
154–165	1480.6772	1480.6749	HGGWHDVGFWQR
166–182	1931.0783	1931.0629	DFELPAPPRPVRPVTQI
Chymotryptic Peptides			
1–27	3037.5157	3037.4862	SPERRPVEIRPATAADMAAVCDIVNHY
36–45	1270.5988	1270.5942	RTEPQTPQEW
36–52	2125.0628	2125.0440	RTEPQTPQEWIDDLERL
46–52	872.4672	872.4603	IDDLERL
46–58	1717.8520	1717.8424	IDDLERLQDRYPW
59–72	1388.6774	1388.7551	LVAEVEGVVAGIAY
77–84	1022.4989	1022.4933	KARNAYDW
77–91	1801.8728	1801.8635	KARNAYDWTVESTVY
83–91	1098.4952	1098.4870	DWTVESTVY
92–99	1031.5795	1031.5737	VSHRHQRL
107–118	1360.6909	1360.6809	THLLKSMEAQGF
110–118	1009.4963	1009.4902	LKSMEAQGF
111–118	896.4103	896.4062	KSMEAQGF
119–135	1763.0467	1763.0305	KSVVAVIGLPNDPSVRL
119–139	2213.2636	2213.2532	KSVVAVIGLPNDPSVRLHEAL
119–141	2433.2604	2433.3380	KSVVAVIGLPNDPSVRLHEALGY
153–162	1138.5356	1138.5309	KHGGWHDVGF
153–163	1324.6190	1324.6102	KHGGWHDVGFW
164–182	2215.2400	2215.2226	QRDFELPAPPRPVRPVTQI
168–182	1668.9828	1668.9675	ELPAPPRPVRPVTQI

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

Table 16. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived PAT Protein Using LC-MS Analysis

Protease	% Coverage	Combined % Coverage
Trypsin	91	95.6
Chymotrypsin	83	

```
  1   SPERRPVEIR  PATAADMAAV  CDIVNHYIET  STVNFRTEPQ  TPQEWIDDLE
 51   RLQDRYPWLV  AEVEGVVAGI  AYAGPWKARN  AYDWTVESTV  YVSHRHQRLG
101   LGSTLYTHLL  KSMEAQGFKS  VVAVIGLPND  PSVRLHEALG  YTARGTLRAA
151   GYKHGGWHDV  GFWQRDFELP  APPRPVRPVT  QI
```

Red/shaded type	Red bold type indicates DP51291 maize-derived PAT peptides identified using LC-MS analysis against the expected PAT protein sequence.
Amino acid residue abbreviations	alanine (A), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), leucine (L), methionine (M), asparagine (N), proline (P), glutamine (Q), arginine (R), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Note: The expected PAT protein sequence does not include the N-terminal methionine as it is anticipated to be absent.

Figure 35. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DP51291 Maize-Derived PAT Protein Using LC-MS Analysis

N-Terminal Amino Acid Sequence Analysis

Samples of the PAT protein purified from DP51291 maize whole plant tissue were analyzed by SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membrane. Protein bands were stained using GelCode Blue stain reagent, and the band containing IPD072Aa protein was excised. The excised band was analyzed using Edman sequencing to determine the N-terminal amino acid sequence.

The analysis identified a sequence (SPERRPVEIR) matching amino acid residues 1-10 of the expected DP51291 maize-derived PAT protein sequence (Table 17), indicating the N-terminal methionine was absent as expected (Dummitt et al., 2003; Sherman et al., 1985).

Additional details regarding N-terminal amino acid sequencing analytical methods are provided in Appendix E.

Table 17. N-Terminal Amino Acid Sequence Analysis of DP51291 Maize-Derived PAT Protein

Expected PAT Sequence	S – P – E – R – R – P – V – E – I – R
Detected PAT Sequence	S – P – E – R – R – P – V – E – I – R

Note: The expected PAT sequence does not include the N-terminal methionine as it is anticipated to be absent.

Allergenicity and Toxicity Analyses of the PAT Protein

The PAT protein present in DP51291 maize is found in several approved events that are currently in commercial use as well as in the corn line DP23211 referenced before. Therefore, in accordance with the FSANZ Application Handbook, only the updated bioinformatics analysis is provided in this dossier for safety assessment.

Bioinformatic Analysis of PAT Protein Homology to Known or Putative Allergens and Toxins ██████, 2022 (PHI-2022-168/225 study))

A bioinformatics assessment of translated stop codon-bracketed frames was conducted according to relevant guidelines (Codex Alimentarius Commission, 2009; FAO/WHO, 2001). All translated stop codon-bracketed frames of length ≥ eight amino acids in the maize (*Zea mays* L.) event DP-Ø51291-2 (referred to as DP51291 maize) sequence that are within the insertion or that cross the boundary between the insertion and its genomic borders were

identified and evaluated. A full description is located in , 2022 (PHI-2022-168/225 study). This report includes the bioinformatic analysis for PAT protein coding sequence (DP51291_233). For information on the PAT protein coding sequence alignments, please refer to Table 3, specifically Frame DP51291_233 in 2022 (PHI-2022-168/225 study). In conclusion, bioinformatics evaluation of the DP51291 insert did not generate biologically relevant amino acid sequence similarities to known allergens, toxins, or other proteins that would be harmful to humans or animals.

Conclusions on the Safety of PAT protein in DP51291 Maize

The amino acid sequence of the PAT protein present in DP51291 maize was demonstrated to be identical to the corresponding protein found in a number of authorized GM events that are currently in commercial use. Protein characterisation results via SDS-PAGE, western blot, peptide mapping, N-terminal amino acid sequence, and glycoprotein analysis have demonstrated that the PAT protein derived from DP51291 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation. The PAT protein has been risk-assessed in previously authorized maize events and is unlikely to present significant risks to the environment, human, or animal health. The updated bioinformatic analyses confirmed lack of any significant amino acid sequence similarity to known and putative toxins and allergens. Based on this weight of evidence, consumption of the PAT protein is unlikely to cause an adverse effect on humans or animals.

PMI protein

Amino Acid Sequence

The gene encoding the PMI protein in DP51291 maize, referred to as the *pmi* gene, was isolated from *Escherichia coli.* PMI served as a selectable marker during transformation which allowed for tissue growth using mannose as the carbon source. The deduced amino acid sequence from translation of the *pmi* gene is 391 amino acids in length and has a molecular weight of approximately 43 kDa (Figure 38; 2018).

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

Figure 36. Deduced Amino Acid Sequence of the PMI Protein
The deduced amino acid sequence from the translation of the *pmi* gene from plasmid The asterisk (*) indicates the translational stop codon. The full-length protein is 391 amino acids in length and has a molecular weight of approximately 43 kDa.

Function and Activity of the PMI Protein

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

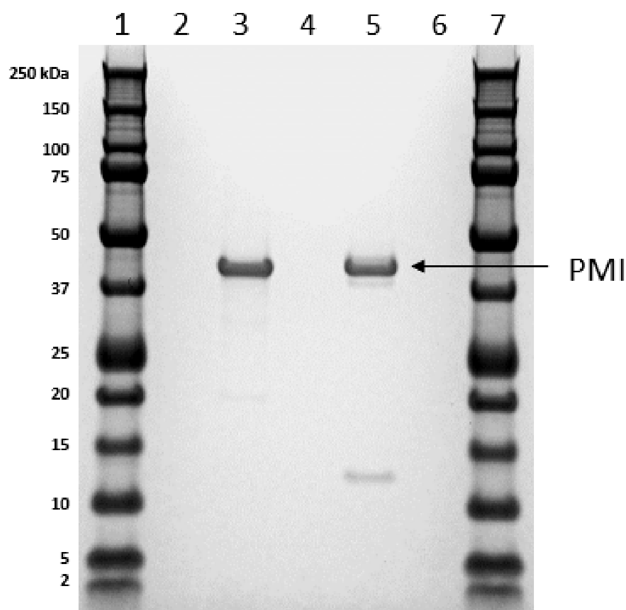
Characterisation of the PMI Protein Derived from DP51291 Maize

The DP51291 maize-expressed PMI protein was characterized using SDS-PAGE analysis, western blot analysis, peptide mapping by mass spectrometry, N-terminal amino acid sequencing, and glycoprotein analysis 2019). The results demonstrated that the PMI protein derived from DP51291 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation.

SDS-PAGE Analysis

Samples of the PMI protein purified from DP51291 maize whole plant tissue were analyzed by SDS-PAGE. As expected, the DP51291 maize derived PMI protein migrated as a predominant band consistent with the expected molecular weight of approximately 43 kDa and a PMI protein reference substance (Figure 37).

Additional details regarding SDS-PAGE analytical methods are provided in Appendix F.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	DP51291 Maize-Derived PMI Protein
4	1X LDS/DTT Sample Buffer Blank
5	Microbially Derived PMI Protein[b]
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

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

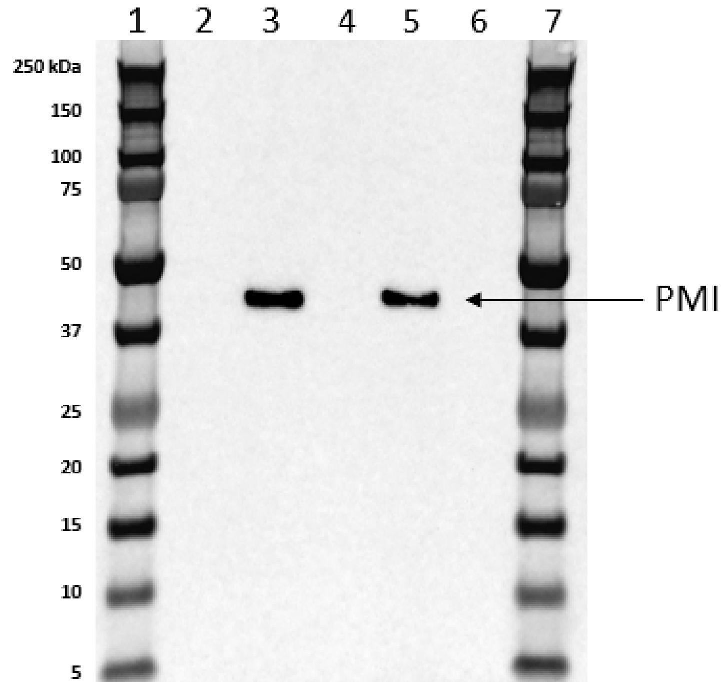
[b] Diluted to 1 µg.

Figure 37. SDS-PAGE Analysis of DP51291 Maize-Derived PMI Protein

Western Blot Analysis

Samples of the PMI protein purified from DP51291 maize whole plant tissue were analyzed by western blot. As expected, the DP51291 maize-derived PMI was immunoreactive to a PMI monoclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 43 kDa and a PMI protein reference substance (Figure 38).

Additional details regarding western blot analytical methods are provided in Appendix F.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	DP51291 Maize-Derived PMI Protein
4	1X LDS/DTT Sample Buffer Blank
5	Microbially Derived PMI Protein[b]
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

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

[b] Diluted to 10 ng.

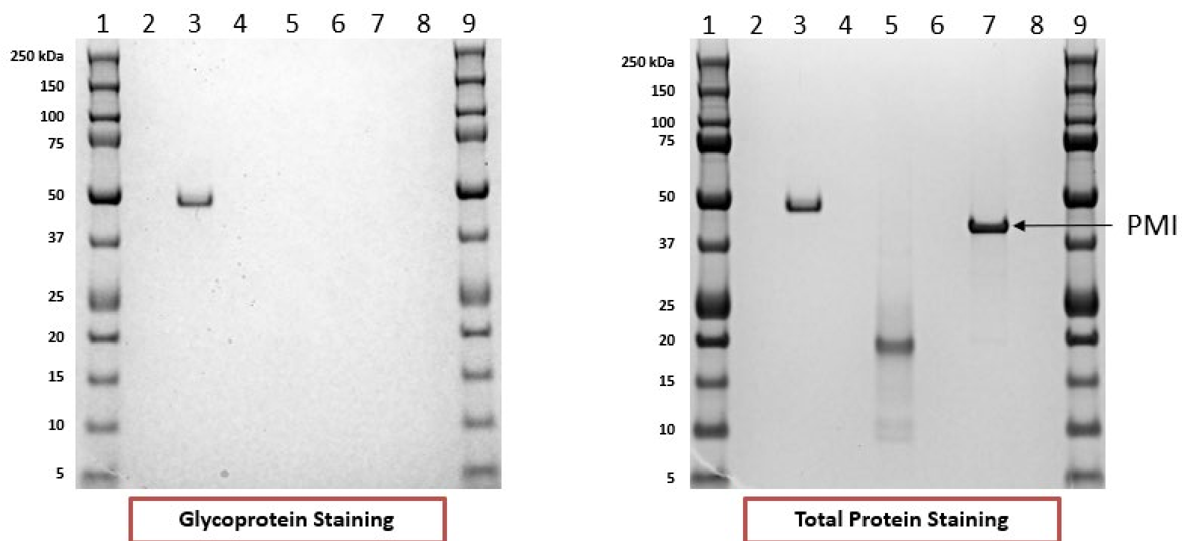
Figure 38. Western Blot Analysis of DP51291 Maize-Derived PMI Protein

Glycosylation Analysis

Samples of the DP51291 maize- derived PMI protein were analyzed by SDS-PAGE for glycosylation analysis. Each gel also included a positive control (horseradish peroxidase) and a negative control (soybean trypsin inhibitor). The gels were stained using a Pierce Glycoprotein Staining Kit to visualize any glycoproteins. The gels were imaged and then stained with Coomassie based GelCode Blue Stain reagent to visualize all protein bands.

Glycosylation was not detected for the DP51291 maize-derived (Figure 39). The horseradish peroxidase positive control was stained and clearly visible as a magenta-colored band. The soybean trypsin inhibitor negative control was not stained by the glycoprotein stain.

Additional details regarding glycosylation analytical methods are provided in Appendix F.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	Horseradish Peroxidase (1.0 µg)
4	1X LDS/DTT Sample Buffer Blank
5	Soybean Trypsin Inhibitor (1.0 µg)
6	1X LDS/DTT Sample Buffer Blank
7	DP51291 Maize-Derived PMI Protein
8	1X LDS/DTT Sample Buffer Blank
9	Pre-stained Protein Molecular Weight Marker[a]

Note: The glycoprotein gel was stained with glycoprotein staining reagent. The total protein stain gel was stained with glycoprotein staining reagent followed by staining with Coomassie blue reagent for total proteins. Kilodalton (kDa) and lithium dodecyl sulfate containing dithiothreitol (LDS/DTT).

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

Figure 39. Glycosylation Analysis of DP51291 Maize-Derived PMI Protein

LC-MS Peptide Mapping and N-Terminal Amino Acid Sequencing Analyses

Samples of the PMI protein purified from DP51291 maize whole plant tissue were analyzed by SDS-PAGE. Protein bands were stained with Coomassie stain reagent, and the band containing PMI protein was excised for each sample. The excised PMI protein bands were digested with trypsin or chymotrypsin. Digested samples were analyzed using LC-MS, and an MS/MS ion search was used to match the detected peaks to peptides from the expected PMI protein sequence.

The identified tryptic and chymotrypic peptides for DP51291 maize-derived PMI protein are shown in Table 18 and Table 19, respectively. The combined sequence coverage was 97.4% (381/391) of the expected PMI amino acid sequence (Table 20 and Figure 40. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis
).

The N-terminal peptide was identified with the LC-MS as MQKLINSVQNY from the chymotryptic digestion. The results indicated the N-terminal methionine residue of the protein was acetylated.

Additional details regarding peptide mapping and N-terminal amino acid sequencing analytical methods are provided in Appendix F.

Table 18. Identified Tryptic Peptides of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
4–16	1478.7082	1478.7518	LINSVQNYAWGSK
17–43	2988.4232	2988.3819	TALTELYGMENPSSQPMAELWMGAHPK
48–59	1241.6863	1241.6728	VQNAAGDIVSLR
48–66	2028.0842	2028.0487	VQNAAGDIVSLRDVIESDK
60–76	1773.8442	1773.936	DVIESDKSTLLGEAVAK
67–76	987.5701	987.56	STLLGEAVAK
67–77	1143.6713	1143.6611	STLLGEAVAKR
77–86	1252.7211	1252.6968	RFGELPFLFK
78–86	1096.5014	1096.5957	FGELPFLFK
87–102	1773.9775	1773.956	VLCAAQPLSIQVHPNK
87–111	2757.4774	2757.4384	VLCAAQPLSIQVHPNKHNSEIGFAK
103–111	1001.5025	1001.493	HNSEIGFAK
112–124	1343.6227	1343.6139	ENAAGIPMDAAER
125–149	2932.5639	2932.5058	NYKDPNHKPELVFALTPFLAMNAFR
128–149	2543.3606	2543.2995	DPNHKPELVFALTPFLAMNAFR[c]
150–179	3269.5996	3269.6833	EFSEIVSLLQPVAGAHPAIAHFLQQPDAER
180–195	1807.8292	1807.9026	LSELFASLLNMQGEEK
204–218	1714.8443	1714.8275	SALDSQQGEPWQTIR
219–240	2479.3773	2479.325	LISEFYPEDSGLFSPLLLNVVK
241–274	3716.9086	3716.8331	LNPGEAMFLFAETPHAYLQGVALEVMANSDNVLR
281–292	1372.7811	1372.7602	YIDIPELVANVK
281–307	3037.7348	3037.6852	YIDIPELVANVKFEAKPANQLLTQPVK
293–307	1682.9558	1682.9355	FEAKPANQLLTQPVK
308–331	2675.3326	2675.2755	QGAELDFPIPVDDFAFSLHDLSDK
332–354	2567.3118	2567.2578	ETTISQQSAAILFCVEGDATLWK
355–379	2598.3989	2598.3653	GSQQLQLKPGESAFIAANESPVTVK

[a] The experimental mass is the uncharged mass calculated from the mass to charge ratio of the observed ion.
[b] The theoretical mass is the *in silico* generated mass that matches most closely to the experimental mass.
[c] This peptide was modified by methionine oxidation.

Table 19. Identified Chymotryptic Peptides of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
1–11	1378.7434	1378.6915	MQKLINSVQNY[c]
12–22	1175.6592	1175.6186	AWGSKTALTEL
14–23	1081.6026	1081.5655	GSKTALTELY
24–36	1389.6351	1389.5904	GMENPSSQPMAEL
24–37	1575.7258	1575.6697	GMENPSSQPMAELW
59–69	1261.6868	1261.6514	RDVIESDKSTL
59–70	1374.7779	1374.7354	RDVIESDKSTLL
59–78	2233.2776	2233.2066	RDVIESDKSTLLGEAVAKRF
70–78	989.5943	989.5658	LGEAVAKRF
70–83	1532.8904	1532.8351	LGEAVAKRFGELPF
71–78	876.5051	876.4817	GEAVAKRF
71–83	1419.8005	1419.751	GEAVAKRFGELPF
95–109	1705.7956	1705.8536	SIQVHPNKHNSEIGF

10–126	1819.9069	1819.8522	AKENAAGIPMDAAERNY
127–137	1322.6556	1322.6983	KDPNHKPELVF
143–151	1113.5635	1113.5277	LAMNAFREF[d]
144–151	984.4801	984.4487	AMNAFREF
149–157	1078.6026	1078.5659	REFSEIVSL
149–158	1191.6931	1191.6499	REFSEIVSLL
152–171	2056.1876	2056.1106	SEIVSLLQPVAGAHPAIAHF
158–171	1427.7179	1427.7674	LQPVAGAHPAIAHF
159–171	1314.6556	1314.6833	QPVAGAHPAIAHF
172–183	1397.7604	1397.715	LQQPDAERLSEL
173–184	1431.7463	1431.6994	QQPDAERLSELF
185–199	1645.9011	1645.8457	ASLLNMQGEEKSRAL
185–199	1661.8925	1661.8406	ASLLNMQGEEKSRAL[d]
188–199	1374.7225	1374.6925	LNMQGEEKSRAL
189–199	1261.6449	1261.6084	NMQGEEKSRAL
189–202	1558.8586	1558.8137	NMQGEEKSRALAIL
200–214	1641.8575	1641.8362	AILKSALDSQQGEPW
203–214	1344.671	1344.631	KSALDSQQGEPW
215–223	1105.6504	1105.6131	QTIRLISEF
235–241	797.5651	797.5375	LLNVVKL
236–248	1430.8083	1430.7592	LNVVKLNPGEAMF
237–248	1317.7202	1317.6751	NVVKLNPGEAMF
237–249	1430.8081	1430.7592	NVVKLNPGEAMFL
249–257	1047.5359	1047.5025	LFAETPHAY
250–257	934.4461	934.4185	FAETPHAY
251–257	787.3727	787.3501	AETPHAY
251–258	900.4647	900.4341	AETPHAYL
258–273	1671.9058	1671.8502	LQGVALEVMANSDNVL
259–273	1558.8332	1558.7661	QGVALEVMANSDNVL
264–273	1090.5249	1090.4965	EVMANSDNVL
278–293	1846.0813	1846.024	TPKYIDIPELVANVKF
282–293	1356.8113	1356.7653	IDIPELVANVKF
294–302	982.5763	982.5447	EAKPANQLL
294–312	2034.1803	2034.1109	EAKPANQLLTQPVKQGAEL
302–312	1182.6929	1182.6608	LTQPVKQGAEL
302–321	2228.219	2228.1365	LTQPVKQGAELDFPIPVDDF
303–312	1069.6088	1069.5768	TQPVKQGAEL
303–321	2115.1319	2115.0525	TQPVKQGAELDFPIPVDDF
303–323	2333.1326	2333.158	TQPVKQGAELDFPIPVDDFAF
313–323	1281.6401	1281.5918	DFPIPVDDFAF
324–343	2156.1703	2156.0961	SLHDLSDKETTISQQSAAIL
329–344	1737.9429	1737.8785	SDKETTISQQSAAILF
354–368	1616.8333	1616.8522	KGSQQLQLKPGESAF
360–368	975.5319	975.5025	QLKPGESAF
369–384	1647.9542	1647.9056	IAANESPVTVKGHGRL
369–388	2137.238	2137.1756	IAANESPVTVKGHGRLARVY

[a] The experimental mass is the uncharged mass calculated from the mass to charge ratio of the observed ion.
[b] The theoretical mass is the *in silico* generated mass that matches most closely to the experimental mass.
[c] The N-terminal peptide was acetylated.
[d] This peptide was modified by methionine oxidation.

Table 20. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis

Protease	% Coverage	Combined % Sequence Coverage
Trypsin	91	97.4
Chymotrypsin	83	

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

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

Figure 40. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DP51291 Maize-Derived PMI Protein Using LC-MS Analysis

Allergenicity and Toxicity Analyses of the PMI Protein

The PMI protein present in DP51291 maize is found in several approved events that are currently in commercial use as well as in the corn line DP23211 referenced before. Therefore, in accordance with the FSANZ Application Handbook, only the updated bioinformatics analysis is provided in this dossier for safety assessment.

Bioinformatic Analysis of PMI Protein Homology to Known or Putative Allergens and Toxins ██████ 2022 (PHI-2022-168/225 study))

A bioinformatics assessment of translated stop codon-bracketed frames was conducted according to relevant guidelines (Codex Alimentarius Commission, 2009; FAO/WHO, 2001). All translated stop codon-bracketed frames of length ≥ eight amino acids in the maize (*Zea mays* L.) event DP-Ø51291-2 (referred to as DP51291 maize) sequence that are within the insertion or that cross the boundary between the insertion and its genomic borders were identified and evaluated. A full description is located in ██████ 2022 (PHI-2022-168/225 study). This report includes the bioinformatic analysis for PMI protein coding sequence (DP51291_110). For information on the PMI protein coding sequence alignments, please refer to Table 3, specifically Frame DP51291_110 in ██████ 2022 (PHI-2022-168/225 study). In conclusion, bioinformatics evaluation of the DP51291 insert did not generate biologically relevant amino acid sequence similarities to known allergens, toxins, or other proteins that would be harmful to humans or animals.

Conclusions on the Safety of PMI Protein in DP51291 Maize

The amino acid sequence of the PMI protein present in DP51291 maize was demonstrated to be identical to the corresponding protein found in a number of authorized GM events that are currently in commercial use. The PMI protein has been risk-assessed in previously authorized maize events and is unlikely to present significant risks to the environment, human, or animal health. The updated bioinformatic analyses confirmed lack of any significant amino acid sequence similarity to known and putative toxins and allergens. Protein characterisation results via SDS-PAGE, western blot, peptide mapping, N-terminal amino acid sequence, and glycoprotein analysis have demonstrated that the PMI protein derived from DP51291 maize is of the expected molecular weight, immunoreactivity, amino acid sequence, and showed a lack of glycosylation. Based on this weight of evidence, consumption of the PMI protein is unlikely to cause an adverse effect on humans or animals.

B.3 OTHER (NON-PROTEIN) NEW SUBSTANCES

There are no other new substances associated with DP51291 maize.

B.4 NOVEL HERBICIDE METABOLITES IN GM HERBICIDE-TOLERANT PLANTS

There are no novel herbicide metabolites associated with DP51291 maize.

B.5 COMPOSITIONAL ANALYSES

Trait expression assessment

The concentration the IPD072Aa, PAT, and PMI proteins were evaluated in DP51291 maize [redacted] 2022 (PHI-2021-034 study)).

Tissue samples were collected during the 2021 growing season at six sites in commercial maize-growing regions of the United States and Canada. A randomized complete block design with four blocks was utilized at each site. The following tissue samples were collected: leaf (V9, R1, and R4 growth stages), root (V6, V9, R1, and R4 growth stages), pollen (R1 growth stage), forage (R4 growth stage), and grain (R6 growth stage). The concentrations of the IPD072Aa, PAT, and PMI proteins were determined using quantitative enzyme linked immunosorbent assays (ELISAs).

Concentration results (means, ranges, and standard deviations) are summarized across sites in Table 21 through Table 23 for IPD072Aa protein, PAT protein, and PMI protein, respectively. Individual sample results below the LLOQ were assigned a value equal to half of the LLOQ for calculation purposes.

Additional details regarding analytical methods and calculations for trait expression analysis are provided in Appendix G.

Table 21. Across-Site Summary of IPD072Aa Protein Concentrations in DP51291 Maize

Tissue (Growth Stage)	ng IPD072Aa/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	Range	Standard Deviation	Sample LLOQ[a]	
DP51291 Maize					
Root (V6)	76	17 - 140	31	0.11	0/24
Root (V9)	140	63 - 230	51	0.11	0/24
Root (R1)	180	66 - 330	85	0.11	0/24
Root (R4)	140	36 - 280	80	0.11	0/23[b]
Leaf (V9)	69	23 - 140	33	0.054	0/24
Leaf (R1)	68	31 - 110	25	0.054	0/24
Leaf (R4)	53	18 - 120	28	0.054	0/24
Pollen (R1)	1.2	0.25 - 7.1	1.4	0.11	0/24
Forage (R4)	34	9.2 - 88	20	0.018	0/24
Grain (R6)	4.1	0.051 - 12	3.6	0.027	0/24
Herbicide-Treated DP51291 Maize					
Root (V6)	67	28 - 120	26	0.11	0/24
Root (V9)	130	33 - 260	61	0.11	0/24
Root (R1)	200	63 - 330	88	0.11	0/24
Root (R4)	140	39 - 300	82	0.11	0/24
Leaf (V9)	68	18 - 130	31	0.054	0/24
Leaf (R1)	68	22 - 120	28	0.054	0/24
Leaf (R4)	52	14 - 90	22	0.054	0/24
Pollen (R1)	1.0	0.35 - 2.5	0.62	0.11	0/24
Forage (R4)	33	9.8 - 62	17	0.018	0/24
Grain (R6)	3.8	0.24 - 11	3.2	0.027	0/24

Note: Growth stages (Abendroth et al., 2011). Herbicide-treated refers to treatment with glufosinate.

[a] Lower limit of quantification (LLOQ) in ng/mg tissue dry weight.

[b] One root sample from one site was not analyzed for IPD072Aa protein due to insufficient quantity. This sample was analyzed for PAT and PMI protein concentrations.

Table 22. Across-Site Summary of PAT Protein Concentrations in DP51291 Maize

Tissue (Growth Stage)	ng PAT/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	Range	Standard Deviation	Sample LLOQ[a]	
DP51291 Maize					
Root (V6)	34	16 - 51	8.0	0.054	0/24
Root (V9)	26	14 - 36	6.2	0.054	0/24
Root (R1)	21	15 - 33	4.6	0.054	0/24
Root (R4)	10	6.6 - 17	2.3	0.054	0/24
Leaf (V9)	38	30 - 49	5.9	0.11	0/24
Leaf (R1)	40	32 - 50	4.5	0.11	0/24
Leaf (R4)	21	15 - 28	3.2	0.11	0/24
Pollen (R1)	67	58 - 83	7.5	0.22	0/24
Forage (R4)	15	11 - 22	2.7	0.036	0/24
Grain (R6)	5.7	2.3 - 9.0	1.8	0.054	0/24
Herbicide-Treated DP51291 Maize					
Root (V6)	33	23 - 42	5.9	0.054	0/24
Root (V9)	26	12 - 42	8.1	0.054	0/24
Root (R1)	20	9.9 - 28	4.4	0.054	0/24
Root (R4)	12	7.5 - 21	3.7	0.054	0/24
Leaf (V9)	37	23 - 47	6.5	0.11	0/24
Leaf (R1)	42	29 - 55	6.3	0.11	0/24
Leaf (R4)	22	16 - 28	2.9	0.11	0/24

Tissue (Growth Stage)	ng PAT/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	Range	Standard Deviation	Sample LLOQ[a]	
Pollen (R1)	68	59 - 80	5.5	0.22	0/24
Forage (R4)	16	11 - 22	2.6	0.036	0/24
Grain (R6)	5.8	3.3 - 11	1.7	0.054	0/24

Note: Growth stages (Abendroth et al., 2011). Herbicide-treated refers to treatment with glufosinate.
[a] Lower limit of quantification (LLOQ) in ng/mg tissue dry weight.

Table 23. Across-Site Summary of PMI Protein Concentrations in DP51291 Maize

Tissue (Growth Stage)	ng PMI/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	Range	Standard Deviation	Sample LLOQ[a]	
DP51291 Maize					
Root (V6)	8.3	2.7 - 13	2.7	0.27	0/24
Root (V9)	6.9	2.9 - 12	2.3	0.27	0/24
Root (R1)	4.8	2.3 - 8.4	1.6	0.27	0/24
Root (R4)	3.7	2.4 - 6.3	1.0	0.27	0/24
Leaf (V9)	8.9	4.4 - 14	2.7	0.54	0/24
Leaf (R1)	13	7.2 - 26	4.6	0.54	0/24
Leaf (R4)	29	17 - 43	6.0	0.54	0/24
Pollen (R1)	29	19 - 37	4.4	1.1	0/24
Forage (R4)	9.2	6.8 - 12	1.3	1.8	0/24
Grain (R6)	4.1	1.7 - 9.3	1.7	0.27	0/24
Herbicide-Treated DP51291 Maize					
Root (V6)	7.3	4.5 - 11	2.3	0.27	0/24
Root (V9)	6.9	2.8 - 11	2.0	0.27	0/24
Root (R1)	4.9	3.3 - 7.5	1.3	0.27	0/24
Root (R4)	3.9	2.3 - 6.0	1.1	0.27	0/24
Leaf (V9)	8.3	4.6 - 14	3.1	0.54	0/24
Leaf (R1)	15	7.8 - 28	5.2	0.54	0/24
Leaf (R4)	31	23 - 38	4.4	0.54	0/24
Pollen (R1)	30	22 - 37	4.3	1.1	0/24
Forage (R4)	9.2	6.2 - 13	1.4	1.8	0/24
Grain (R6)	3.7	2.0 - 5.7	0.94	0.27	0/24

Note: Growth stages (Abendroth et al., 2011). Herbicide-treated refers to treatment with glufosinate.
[a] Lower limit of quantification (LLOQ) in ng/mg tissue dry weight.

Nutrient composition assessment

An assessment of the compositional equivalence of a GM product compared to that of a conventional non-GM comparator with a history of safe use in food and feed is an important part of the weight-of-evidence approach used to evaluate the safety of genetically modified plant products (Codex Alimentarius Commission, 2008; OECD, 1993). Compositional assessments of DP51291 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 biological variation established from multiple sources of non-GM, commercial maize data (reference maize) ██████████, 2022 (PHI-2021-035/021 study)).

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected during the 2021 growing season at eight sites in commercial maize-growing regions of the United States and Canada. A randomized complete block design with four blocks was utilized at each site. Each block included DP51291 maize, non-GM near-isoline control maize, and four non-GM commercial maize reference lines. An herbicide treatment of glufosinate was applied to DP51291 maize.

The samples were assessed for key nutritional components. Proximate, fiber, and mineral analytes were assessed in the forage samples (9 analytes total), and grain samples were assessed for proximate, fiber, fatty acid, amino acid, mineral, vitamin, secondary metabolite, and anti-nutrient analytes (70 analytes total). The analytes included in the compositional assessment were selected based on the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002). 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 were validated methods, with the majority based on methods published by AOAC International, AACC (American Association of Cereal Chemists), and AOCS (American Oil Chemists' Society).

Statistical analyses were conducted to evaluate and compare the nutrient composition of DP51291 maize and the control maize. Across-site comparisons were conducted for a total of 79 analytes, where 68 analytes were analyzed using mixed model analysis and 5 analytes did not meet criteria for sufficient quantities of observations above the LLOQ and were therefore subjected to Fisher's exact test. No statistical analysis was conducted on the remaining 6 analytes as all data values were below the LLOQ. For a given analyte in the mixed model analysis, if a statistical difference (P-value < 0.05) was observed between DP951291 maize and the control maize, the False Discovery Rate (FDR)-adjusted P-value was examined. In cases where the raw P-value indicated a significant difference but the FDR-adjusted P-value was non-significant, it was concluded that the difference was likely a false positive. Additionally, three reference ranges representing the non-GM maize population with a history of safe use (i.e., tolerance interval, literature range, and in-study reference range) were utilized to evaluate statistical differences in the context of biological variation. If the measured values of DP51291 maize for that analyte fell within at least one of the reference ranges, then this analyte would be considered comparable to conventional maize.

The outcome of the nutrient composition assessment is provided in Table 24. Nutrient composition analysis results are provided in Tables Table 25 to Table 31. No statistically significant differences were identified between herbicide-treated DP51291 maize and the control maize for 64 of the 73 analytes evaluated in the across-site analysis via either mixed model analysis or Fisher's exact test. A statistically significant difference, before FDR adjustment, was observed in the across-site analysis between DP51291 maize and the control maize for nine analytes (crude protein [forage], carbohydrates [forage], palmitic acid [C16:0], oleic acid [C18:1], linoleic acid [C18:2], eicosenoic acid [C20:1], lignoceric acid [C24:0], copper, and trypsin inhibitor). Although raw P-values were significant for carbohydrates

(forage), palmitic acid (C16:0), linoleic acid (C18:2), eicosenoic acid (C20:1), lignoceric acid (C24:0), copper, and trypsin inhibitor, the FDR adjusted P-value was non-significant, indicating that the identified statistical differences were likely false positives. Additionally, for all nine of these analytes, the range of values for herbicide-treated DP51291 maize were within one or more of the reference ranges (i.e., tolerance interval, literature range, and in-study reference range) representing the non-GM maize population with a history of safe use, indicating that herbicide-treated DP51291 maize is within the range of normal variation for these analytes and the statistical difference is not biologically meaningful.

The results of the nutrient composition assessment demonstrated that nutrient composition of forage and grain derived from DP51291 maize was comparable to that of conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Additional details regarding methods for nutrient composition and statistical analyses are provided in Appendix H.

Table 24. Outcome of Across-Site Nutrient Composition Assessment for DP51291 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			
				All Data Values Within Reference Data Range	One or More Data Values Outside Reference Data Range		
Forage (R4 Growth Stage)							
Proximate, Fiber, and Mineral Composition	Crude Fat Crude Fiber ADF NDF Ash Calcium Phosphorus	Crude Protein Carbohydrates	--	--	--	Crude Protein	--
Grain (R6 Growth Stage)							
Proximate and Fiber Composition	Moisture (%) Crude Protein Crude Fat Crude Fiber ADF NDF Total Dietary Fiber Ash Carbohydrates	--	--	--	--	--	--

Table 25. Outcome of the Nutrient Composition Assessment for DP51291 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			
				All Data Values Within Reference Data Range	One or More Data Values Outside Reference Data Range		
Grain (R6 Growth Stage)							
Fatty Acid Composition	Palmitoleic Acid (C16:1) Heptadecanoic Acid (C17:0) Stearic Acid (C18:0) α-Linolenic Acid (C18:3) Arachidic Acid (C20:0) Behenic Acid (C22:0)	Palmitic Acid (C16:0) Oleic Acid (C18:1) Linoleic Acid (C18:2) Eicosenoic Acid (C20:1) Lignoceric Acid (C24:0)	--	--	--	Oleic Acid (C18:1)	Lauric Acid (C12:0) Myristic Acid (C14:0) Heptadecenoic Acid (C17:1) Eicosadienoic Acid (C20:2)
Amino Acid Composition	Alanine Arginine Aspartic Acid Cystine Glutamic Acid Glycine Histidine Isoleucine Leucine Lysine Methionine Phenylalanine Proline Serine Threonine Tryptophan Tyrosine Valine	--	--	--	--	--	--

Table 25. Outcome of the Nutrient Composition Assessment for DP51291 Maize(continued)

<table>
<tr><th rowspan="4">Subgroup</th><th rowspan="4">No Statistical Difference Identified</th><th colspan="5">Statistical Difference Identified</th><th rowspan="4">Not Included in Statistical Analysis (All Data Values Below the Lower Limit of Quantification)</th></tr>
<tr><th rowspan="3">All Data Values Within Tolerance Interval</th><th colspan="3">One or More Data Values Outside Tolerance Interval, or Tolerance Interval Not Available</th><th rowspan="3">Adjusted P-Value < 0.05</th></tr>
<tr><th rowspan="2">All Data Values Within Literature Range</th><th colspan="2">One or More Data Values Outside Literature Range</th></tr>
<tr><th>All Data Values Within Reference Data Range</th><th>One or More Data Values Outside Reference Data Range</th></tr>
<tr><td colspan="8">**Grain (R6 Growth Stage)**</td></tr>
<tr><td>Mineral Composition</td><td>Calcium
Iron
Magnesium
Manganese
Phosphorus
Potassium
Sodium
Zinc</td><td>Copper</td><td>--</td><td>--</td><td>--</td><td>--</td><td>--</td></tr>
<tr><td>Vitamin Composition</td><td>β-Carotene
Vitamin B1 (Thiamine)
Vitamin B3 (Niacin)
Vitamin B5 (Pantothenic Acid)
Vitamin B6 (Pyridoxine)
Vitamin B9 (Folic Acid)
α-Tocopherol
β-Tocopherol
γ-Tocopherol
δ-Tocopherol
Total Tocopherols</td><td>--</td><td>--</td><td>--</td><td>--</td><td>--</td><td>Vitamin B2 (Riboflavin)</td></tr>
<tr><td>Secondary Metabolite and Anti-Nutrient Composition</td><td>*p*-Coumaric Acid
Ferulic Acid
Inositol
Phytic Acid
Raffinose</td><td>Trypsin Inhibitor</td><td>--</td><td>--</td><td>--</td><td>--</td><td>Furfural</td></tr>
</table>

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

Proximates, Fiber, and Minerals Assessment of DP51291 Maize Forage

Proximates, fiber, and minerals were analyzed in forage derived from DP51291 maize and control maize. Results are shown in Table 25. No statistically significant differences (P-value < 0.05) were observed between DP51291 maize and control maize, with an exception for two analytes. A statistically significant difference (P-value < 0.05) was observed between DP51291 maize and control maize for crude protein and carbohydrates. All individual values for these analytes were within the respective tolerance interval, indicating DP51291 maize is within the range of biological variation for these analytes and the statistical difference are not biologically meaningful. Additionally, the non-significant FDR-adjusted P-value for carbohydrates indicates that this difference was likely a false positive.

These results demonstrate that the proximate, fiber, and mineral composition of forage derived from DP51291 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 25. Proximates, Fiber, and Minerals Results for DP51291 Maize Forage

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
Crude Protein	Mean	8.13	7.62	3.78 - 12.2	2.37 - 16.32	5.76 - 10.4
	Range	5.29 - 10.5	5.29 - 9.68			
	Confidence Interval	7.30 - 8.95	6.80 - 8.44			
	Adjusted P-Value	--	0.0360†			
	P-Value	--	0.00106*			
Crude Fat	Mean	3.93	3.86	0.920 - 6.55	NQ - 7.500	2.03 - 6.60
	Range	2.53 - 5.80	2.73 - 5.71			
	Confidence Interval	3.54 - 4.31	3.48 - 4.24			
	Adjusted P-Value	--	0.946			
	P-Value	--	0.709			
Crude Fiber	Mean	25.1	24.2	13.8 - 31.0	12.1 - 42	16.8 - 29.3
	Range	19.6 - 30.7	19.5 - 31.2			
	Confidence Interval	24.2 - 25.9	23.3 - 25.1			
	Adjusted P-Value	--	0.559			
	P-Value	--	0.154			
ADF	Mean	29.6	29.0	15.8 - 40.0	5.13 - 47.39	21.0 - 37.7
	Range	24.7 - 38.2	21.4 - 36.1			
	Confidence Interval	28.7 - 30.6	28.0 - 29.9			
	Adjusted P-Value	--	0.697			
	P-Value	--	0.288			
NDF	Mean	51.1	50.6	29.3 - 62.9	18.30 - 67.80	33.7 - 60.2
	Range	42.9 - 58.0	43.9 - 60.2			
	Confidence Interval	49.5 - 52.8	48.9 - 52.2			
	Adjusted P-Value	--	0.834			
	P-Value	--	0.515			
Ash	Mean	4.89	4.64	2.45 - 9.39	0.66 - 13.20	2.95 - 8.97
	Range	2.97 - 6.95	3.13 - 7.33			
	Confidence Interval	3.89 - 5.90	3.63 - 5.65			
	Adjusted P-Value	--	0.540			
	P-Value	--	0.119			
Carbohydrates	Mean	83.0	83.9	76.7 - 91.0	73.3 - 92.9	77.7 - 87.6
	Range	79.3 - 88.0	80.4 - 88.2			
	Confidence Interval	81.4 - 84.6	82.3 - 85.5			
	Adjusted P-Value	--	0.0538			
	P-Value	--	0.00395*			
Calcium	Mean	0.232	0.223	0.0593 - 0.461	0.04 - 0.58	0.136 - 0.430
	Range	0.151 - 0.353	0.139 - 0.312			
	Confidence Interval	0.194 - 0.271	0.185 - 0.262			
	Adjusted P-Value	--	0.834			
	P-Value	--	0.458			
Phosphorus	Mean	0.271	0.256	0.0697 - 0.407	0.07 - 0.55	0.153 - 0.441
	Range	0.158 - 0.418	0.137 - 0.376			
	Confidence Interval	0.224 - 0.318	0.209 - 0.303			

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Adjusted P-Value	--	0.579			
	P-Value	--	0.170			

Note: Note: Not quantified (NQ); one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified. Proximate, Fiber, and Mineral Composition are reported as % Dry Weight.

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

† Adjusted P-Value < 0.05 was observed.

Proximates and Fiber Assessment of DP51291 Maize Grain

Proximates and fiber were analyzed in grain derived from DP51291 maize and control maize. Results are shown in Table 26. No statistically significant differences (P-value < 0.05) were observed between DP51291 maize and control maize.

The results demonstrate that the proximates and fiber of grain derived from DP51291 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 26. Proximates and Fiber Results for DP51291 Maize Grain

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
Moisture (%)	Mean	20.8	20.3			
	Range	10.0 - 30.0	9.56 - 30.1			
	Confidence Interval	15.4 - 26.1	15.0 - 25.7	3.67 - 38.1	5.1 - 40.7	9.34 - 33.9
	Adjusted P-Value	--	0.559			
	P-Value	--	0.142			
Crude Protein	Mean	10.7	10.7			
	Range	9.11 - 12.0	8.64 - 12.2			
	Confidence Interval	10.2 - 11.3	10.1 - 11.2	6.57 - 13.1	5.72 - 17.26	7.19 - 12.7
	Adjusted P-Value	--	0.955			
	P-Value	--	0.772			
Crude Fat	Mean	3.47	3.67			
	Range	2.42 - 4.71	2.80 - 4.68			
	Confidence Interval	3.18 - 3.75	3.39 - 3.96	2.33 - 5.91	1.363 - 7.830	2.37 - 5.41
	Adjusted P-Value	--	0.485			
	P-Value	--	0.100			
Crude Fiber	Mean	2.53	2.49			
	Range	2.03 - 3.00	2.08 - 2.79			
	Confidence Interval	2.41 - 2.66	2.37 - 2.61	1.61 - 3.49	0.49 - 5.5	1.98 - 3.15
	Adjusted P-Value	--	0.697			
	P-Value	--	0.297			
ADF	Mean	4.63	4.56			
	Range	3.98 - 5.38	3.82 - 5.44			
	Confidence Interval	4.46 - 4.80	4.39 - 4.73	2.70 - 6.12	1.41 - 11.34	3.05 - 5.64
	Adjusted P-Value	--	0.834			
	P-Value	--	0.454			
NDF	Mean	10.4	10.4			
	Range	9.32 - 11.3	9.37 - 13.1			
	Confidence Interval	10.1 - 10.6	10.2 - 10.7	7.66 - 17.4	4.28 - 24.30	8.93 - 13.8
	Adjusted P-Value	--	0.946			
	P-Value	--	0.701			
Total Dietary Fiber	Mean	9.78	9.92			
	Range	8.42 - 11.2	7.83 - 11.0			
	Confidence Interval	9.44 - 10.1	9.57 - 10.3	2.98 - 19.4	4.44 - 35.31	7.87 - 12.7
	Adjusted P-Value	--	0.763			
	P-Value	--	0.359			

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
Ash	Mean	1.31	1.32	1.01 - 1.82	0.616 - 6.282	0.971 - 1.67
	Range	1.00 - 1.55	1.04 - 1.58			
	Confidence Interval	1.23 - 1.40	1.23 - 1.40			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.868			
Carbohydrates	Mean	84.5	84.3	80.6 - 88.7	77.4 - 89.7	81.5 - 88.2
	Range	83.0 - 86.6	82.6 - 86.4			
	Confidence Interval	83.9 - 85.1	83.7 - 84.9			
	Adjusted P-Value	--	0.834			
	P-Value	--	0.436			

Note: This table provides results from the mixed model analysis only. Proximate and Fiber Composition are reported as % Dry Weight or as Indicated.

Fatty Acids Assessment of DP51291 Maize Grain

Fatty acids were analyzed in grain derived from DP51291 maize and control maize. Results are shown in Table 27. No statistically significant differences (P-value < 0.05) were observed between DP51291 maize and control maize with an exception for five analytes. A statistically significant difference (P-value < 0.05) was observed between DP51291 maize and control maize for palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), eicosenoic acid (C20:1), and lignoceric acid (C24:0). All individual values for these analytes were within the respective tolerance interval, indicating DP51291 maize is within the range of biological variation for these analytes and the statistical difference are not biologically meaningful. Additionally, the non-significant FDR-adjusted P-value for palmitic acid (C16:0), linoleic acid (C18:2), eicosenoic acid (C20:1), and lignoceric acid (C24:0) indicates that this difference was likely a false positive.

These results demonstrate that the fatty acid composition of grain derived from DP51291 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 27. Fatty Acid Results for DP51291 Maize Grain

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
Lauric Acid (C12:0)	Mean	<LLOQ[a]	<LLOQ[a]			
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA	0 - 0.423[r]	NQ - 0.698	<LLOQ[a]
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Myristic Acid (C14:0)	Mean	<LLOQ[a]	<LLOQ[a]			
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA	0 - 0.267[r]	NQ - 0.288	<LLOQ[a]
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Palmitic Acid (C16:0)	Mean	13.5	13.3			
	Range	12.5 - 14.3	12.6 - 13.9			
	Confidence Interval	13.2 - 13.7	13.1 - 13.6	9.60 - 24.2	6.81 - 39.0	10.3 - 14.3
	Adjusted P-Value	--	0.252			
	P-Value	--	0.0259*			
Palmitoleic Acid (C16:1)	Mean	0.103	0.107			
	Range	<LLOQ[a] - 0.120	<LLOQ[a] - 0.126			
	Confidence Interval	0.0938 - 0.112	0.0976 - 0.115	0 - 0.418	NQ - 0.67	<LLOQ[a] - 0.187
	Adjusted P-Value	--	0.555			
	P-Value	--	0.131			
Heptadecanoic Acid (C17:0)	Mean	0.0494	0.0516			
	Range	<LLOQ[a] - 0.0883	<LLOQ[a] - 0.0887			
	Confidence Interval	NA	NA	0 - 0.221	NQ - 0.203	<LLOQ[a] - 0.0986
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Heptadecenoic Acid (C17:1)	Mean	<LLOQ[a]	<LLOQ[a]			
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA	0 - 0.135[r]	NQ - 0.131	<LLOQ[a]
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Stearic Acid (C18:0)	Mean	1.66	1.67			
	Range	1.35 - 1.95	1.37 - 1.95			
	Confidence Interval	1.51 - 1.81	1.52 - 1.82	1.33 - 3.66	NQ - 4.9	1.18 - 2.54
	Adjusted P-Value	--	0.804			
	P-Value	--	0.390			
Oleic Acid (C18:1)	Mean	21.9	22.5			
	Range	20.4 - 23.6	21.1 - 23.9			
	Confidence Interval	21.3 - 22.6	21.8 - 23.1	19.5 - 38.4	16.38 - 42.81	23.1 - 34.5
	Adjusted P-Value	--	0.000105†			
	P-Value	--	<0.0001*			
Linoleic Acid (C18:2)	Mean	60.0	59.6	33.7 - 65.1	13.1 - 67.68	47.9 - 59.2
	Range	58.6 - 61.7	58.5 - 60.5			

	Confidence Interval	59.5 - 60.5	59.1 - 60.1			
	Adjusted P-Value	--	0.0524			
	P-Value	--	0.00267[*]			
α-Linolenic Acid (C18:3)	Mean	1.56	1.54	0 - 2.12	NQ - 2.33	1.16 - 1.73
	Range	1.33 - 1.66	1.32 - 1.77			
	Confidence Interval	1.51 - 1.61	1.49 - 1.59			
	Adjusted P-Value	--	0.644			
	P-Value	--	0.237			
Arachidic Acid (C20:0)	Mean	0.364	0.360	0.298 - 0.794	0.267 - 1.2	0.276 - 0.529
	Range	0.306 - 0.411	0.310 - 0.408			
	Confidence Interval	0.339 - 0.389	0.335 - 0.385			
	Adjusted P-Value	--	0.452			
	P-Value	--	0.0731			
Eicosenoic Acid (C20:1)	Mean	0.255	0.249	0 - 0.556	NQ - 1.952	0.162 - 0.296
	Range	0.228 - 0.278	0.221 - 0.292			
	Confidence Interval	0.244 - 0.266	0.238 - 0.260			
	Adjusted P-Value	--	0.0524			
	P-Value	--	0.00308[*]			
Eicosadienoic Acid (C20:2)	Mean	<LLOQ[a]	<LLOQ[a]	0 - 0.825[r]	NQ - 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	0.135	0.145	0 - 0.423	NQ - 0.5	<LLOQ[a] - 0.298
	Range	<LLOQ[a] - 0.227	<LLOQ[a] - 0.239			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Lignoceric Acid (C24:0)	Mean	0.262	0.256	0 - 0.605	NQ - 0.91	0.183 - 0.418
	Range	0.216 - 0.328	0.217 - 0.321			
	Confidence Interval	0.238 - 0.286	0.232 - 0.280			
	Adjusted P-Value	--	0.336			
	P-Value	--	0.0445[*]			

Note: This table provides results from the mixed model analysis only. Not applicable (NA); mixed model analysis was not performed, or confidence interval was not determined. Not quantified (NQ); one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified. Fatty acid composition is reported as % total fatty acids.

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

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

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

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

Amino Acids Assessment in DP51291 Maize Grain

Amino acids were analyzed in grain derived from DP51291 maize and control maize. Results are shown in Table 28. No statistically significant differences (P-value < 0.05) were observed between DP51291 maize and control maize.

These results demonstrate that the amino acid composition of grain derived from DP51291 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 28. Amino Acid Results for DP51291 Maize Grain

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
Alanine	Mean	0.812	0.812	0.448 - 1.06	0.40 - 1.48	0.508 - 0.957
	Range	0.637 - 0.994	0.616 - 0.989			
	Confidence Interval	0.758 - 0.866	0.758 - 0.866			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.981			
Arginine	Mean	0.458	0.447	0.299 - 0.586	0.12 - 0.71	0.363 - 0.587
	Range	0.357 - 0.499	0.363 - 0.499			
	Confidence Interval	0.437 - 0.478	0.427 - 0.467			
	Adjusted P-Value	--	0.669			
	P-Value	--	0.266			
Aspartic Acid	Mean	0.658	0.656	0.413 - 0.889	0.30 - 1.21	0.423 - 0.744
	Range	0.519 - 0.810	0.485 - 0.800			
	Confidence Interval	0.615 - 0.700	0.614 - 0.698			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.893			
Cystine	Mean	0.192	0.196	0.125 - 0.294	0.09 - 0.51	0.114 - 0.274
	Range	0.143 - 0.229	0.133 - 0.278			
	Confidence Interval	0.176 - 0.209	0.180 - 0.213			
	Adjusted P-Value	--	0.856			
	P-Value	--	0.541			
Glutamic Acid	Mean	2.14	2.14	1.10 - 2.75	0.83 - 3.54	1.28 - 2.46
	Range	1.68 - 2.60	1.63 - 2.56			
	Confidence Interval	1.99 - 2.28	2.00 - 2.28			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.956			
Glycine	Mean	0.398	0.397	0.285 - 0.482	0.184 - 0.685	0.334 - 0.480
	Range	0.308 - 0.428	0.347 - 0.438			
	Confidence Interval	0.382 - 0.414	0.381 - 0.413			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.919			
Histidine	Mean	0.333	0.332	0.191 - 0.384	0.14 - 0.46	0.244 - 0.371
	Range	0.248 - 0.373	0.280 - 0.368			
	Confidence Interval	0.318 - 0.348	0.317 - 0.348			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.918			
Isoleucine	Mean	0.395	0.398	0.208 - 0.494	0.18 - 0.69	0.265 - 0.443
	Range	0.304 - 0.477	0.312 - 0.479			
	Confidence Interval	0.373 - 0.417	0.376 - 0.421			
	Adjusted P-Value	--	0.932			
	P-Value	--	0.658			
Leucine	Mean	1.43	1.44	0.678 - 1.82	0.60 - 2.49	0.855 - 1.63
	Range	1.12 - 1.79	1.10 - 1.77			

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Confidence Interval	1.33 - 1.53	1.34 - 1.54			
	Adjusted P-Value	--	0.955			
	P-Value	--	0.759			
Lysine	Mean	0.290	0.286	0.182 - 0.395	0.127 - 0.668	0.224 - 0.342
	Range	0.237 - 0.331	0.239 - 0.317			
	Confidence Interval	0.278 - 0.301	0.275 - 0.297			
	Adjusted P-Value	--	0.834			
	P-Value	--	0.510			

Table 30. Amino Acid Results for DP51291 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
Methionine	Mean	0.207	0.212	0.105 - 0.312	0.09 - 0.47	0.146 - 0.281
	Range	0.135 - 0.260	0.156 - 0.276			
	Confidence Interval	0.191 - 0.223	0.196 - 0.228			
	Adjusted P-Value	--	0.834			
	P-Value	--	0.487			
Phenylalanine	Mean	0.599	0.601	0.299 - 0.732	0.24 - 0.93	0.384 - 0.676
	Range	0.469 - 0.741	0.471 - 0.738			
	Confidence Interval	0.559 - 0.639	0.561 - 0.641			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.909			
Proline	Mean	0.999	1.00	0.550 - 1.24	0.46 - 1.75	0.638 - 1.09
	Range	0.779 - 1.17	0.816 - 1.17			
	Confidence Interval	0.942 - 1.06	0.947 - 1.06			
	Adjusted P-Value	--	0.955			
	P-Value	--	0.756			
Serine	Mean	0.546	0.555	0.310 - 0.678	0.15 - 0.91	0.366 - 0.631
	Range	0.413 - 0.631	0.442 - 0.639			
	Confidence Interval	0.512 - 0.580	0.521 - 0.589			
	Adjusted P-Value	--	0.763			
	P-Value	--	0.356			
Threonine	Mean	0.399	0.402	0.250 - 0.484	0.17 - 0.67	0.289 - 0.439
	Range	0.306 - 0.448	0.333 - 0.457			
	Confidence Interval	0.379 - 0.419	0.382 - 0.422			
	Adjusted P-Value	--	0.904			
	P-Value	--	0.625			
Tryptophan	Mean	0.0626	0.0610	0.0375 - 0.0984	0.027 - 0.215	0.0453 - 0.0718
	Range	0.0485 - 0.0731	0.0483 - 0.0717			
	Confidence Interval	0.0598 - 0.0655	0.0582 - 0.0638			
	Adjusted P-Value	--	0.637			
	P-Value	--	0.213			
Tyrosine	Mean	0.307	0.290	0.146 - 0.504	0.10 - 0.73	0.159 - 0.375
	Range	0.207 - 0.405	0.173 - 0.393			
	Confidence Interval	0.277 - 0.338	0.260 - 0.320			
	Adjusted P-Value	--	0.559			
	P-Value	--	0.156			
Valine	Mean	0.505	0.505	0.302 - 0.628	0.21 - 0.86	0.368 - 0.581
	Range	0.386 - 0.573	0.415 - 0.581			
	Confidence Interval	0.482 - 0.528	0.482 - 0.528			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.950			

Note: This table provides results from the mixed model analysis only. Amino acid composition is reported as % Dry Weight.

Minerals Assessment of DP51291 Maize Grain

Minerals were analyzed in grain derived from DP51291 maize and control maize. Results are shown in Table 29. No statistically significant differences (P-value < 0.05) were observed between DP51291 maize and control maize, with an exception for one analyte. A statistically significant difference (P-value < 0.05) was observed between DP51291 maize and control maize for copper. All individual values for this analyte were within the tolerance interval, indicating DP51291 maize is within the range of biological variation for this analyte and the statistical difference is not biologically meaningful. Additionally, the non-significant FDR-adjusted P-value for copper indicates that this difference was likely a false positive.

These results demonstrate that the mineral composition of grain derived from DP51291 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 29. Mineral Results for DP51291 Maize Grain

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
Calcium	Mean	0.00457	0.00461	0.00141 - 0.00731	NQ - 0.101	0.00280 - 0.00861
	Range	0.00324 - 0.00761	0.00332 - 0.00754			
	Confidence Interval	0.00373 - 0.00549	0.00377 - 0.00553			
	Adjusted P-Value	--	0.946			
	P-Value	--	0.690			
Copper	Mean	0.0000982	0.000114	<0.0000625[b] - 0.000341	NQ - 0.0021	<0.0000625[b] - 0.000232
	Range	<0.0000625[b] - 0.000205	<0.0000625[b] - 0.000219			
	Confidence Interval	0.0000593 - 0.000137	0.0000752 - 0.000153			
	Adjusted P-Value	--	0.0625			
	P-Value	--	0.00551*			
Iron	Mean	0.00174	0.00173	0.00112 - 0.00299	0.0000712 - 0.0191	0.00103 - 0.00248
	Range	0.00102 - 0.00217	0.00115 - 0.00208			
	Confidence Interval	0.00151 - 0.00196	0.00151 - 0.00196			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.870			
Magnesium	Mean	0.138	0.136	0.0800 - 0.157	0.0035 - 1.000	0.0876 - 0.153
	Range	0.114 - 0.167	0.109 - 0.166			
	Confidence Interval	0.128 - 0.148	0.126 - 0.145			
	Adjusted P-Value	--	0.669			
	P-Value	--	0.259			
Manganese	Mean	0.000827	0.000800	0.000227 - 0.00105	0.0000312 - 0.0054	0.000379 - 0.000967
	Range	0.000625 - 0.00123	0.000573 - 0.00117			
	Confidence Interval	0.000721 - 0.000934	0.000694 - 0.000906			
	Adjusted P-Value	--	0.485			
	P-Value	--	0.0963			
Phosphorus	Mean	0.357	0.361	0.213 - 0.415	0.010 - 0.750	0.210 - 0.448
	Range	0.270 - 0.457	0.236 - 0.438			
	Confidence Interval	0.326 - 0.389	0.330 - 0.393			
	Adjusted P-Value	--	0.860			
	P-Value	--	0.556			
Potassium	Mean	0.358	0.365	0.239 - 0.502	0.020 - 0.720	0.287 - 0.517
	Range	0.310 - 0.436	0.306 - 0.412			
	Confidence Interval	0.341 - 0.376	0.348 - 0.382			
	Adjusted P-Value	--	0.763			
	P-Value	--	0.348			
Sodium	Mean	0.000260	0.000291	<LLOQ[b] - 0.0133	NQ - 0.15	<0.0000625[b] - 0.00398
	Range	<0.0000625[b] - 0.00235	0.0000721 - 0.00308			
	Confidence Interval	0.000181 - 0.000402	0.000200 - 0.000464			
	Adjusted P-Value	--	0.865			
	P-Value	--	0.572			
Zinc	Mean	0.00217	0.00217	0.00135 - 0.00341	0.0000283 - 0.0043	0.00149 - 0.00356
	Range	0.00162 - 0.00265	0.00161 - 0.00283			

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Confidence Interval	0.00196 - 0.00238	0.00196 - 0.00238			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.958			

Note: This table provides results from the mixed model analysis only. Not quantified (NQ); one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified. Mineral composition is reported as % Dry Weight.

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

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

Vitamin Assessment of DP51291 Maize Grain

Vitamins were analyzed in grain derived from DP51291 maize and control maize. Results are shown in Table 30. No statistically significant differences (P-value < 0.05) were observed between DP51291 maize and control maize.

These results demonstrate that the vitamin composition of grain derived from DP51291 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 30. Vitamin Results for DP51291 Maize Grain

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
β-Carotene	Mean	0.685	0.692			
	Range	0.336 - 1.35	0.248 - 1.44			
	Confidence Interval	0.422 - 0.948	0.429 - 0.955	0 - 3.48	NQ - 5.81	0.201 - 2.01
	Adjusted P-Value	--	0.981			
	P-Value	--	0.848			
Vitamin B1 (Thiamine)	Mean	2.24	2.28			
	Range	1.78 - 2.68	1.83 - 2.88			
	Confidence Interval	2.07 - 2.41	2.11 - 2.45	<LLOQ[b] - 5.16	NQ - 40.00	<0.900[b] - 2.89
	Adjusted P-Value	--	0.834			
	P-Value	--	0.492			
Vitamin B2 (Riboflavin)	Mean	<0.900[b]	<0.900[b]			
	Range	<0.900[b]	<0.900[b]			
	Confidence Interval	NA	NA	<0.900[b] - 2.27[r]	NQ - 7.35	<0.900[b] - 1.49
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Vitamin B3 (Niacin)	Mean	13.8	13.7			
	Range	8.43 - 20.4	8.57 - 24.0			
	Confidence Interval	12.3 - 15.2	12.2 - 15.1	7.91 - 29.3	NQ - 70	8.57 - 24.7
	Adjusted P-Value	--	0.981			
	P-Value	--	0.868			
Vitamin B5 (Pantothenic Acid)	Mean	6.23	6.06			
	Range	4.30 - 7.38	4.32 - 7.19			
	Confidence Interval	5.64 - 6.81	5.48 - 6.65	2.61 - 7.62	2.40 - 14	4.52 - 7.74
	Adjusted P-Value	--	0.485			
	P-Value	--	0.0886			
Vitamin B6 (Pyridoxine)	Mean	4.76	4.55			
	Range	2.08 - 7.60	1.83 - 7.91			
	Confidence Interval	3.88 - 5.65	3.66 - 5.44	0.982 - 8.95	NQ - 12.14	2.13 - 9.88
	Adjusted P-Value	--	0.595			
	P-Value	--	0.184			
Vitamin B9 (Folic Acid)	Mean	4.34	3.86			
	Range	2.01 - 9.93	1.64 - 9.69			
	Confidence Interval	3.29 - 5.73	2.93 - 5.09	0.219 - 6.16	NQ - 11.40	1.93 - 9.75
	Adjusted P-Value	--	0.637			
	P-Value	--	0.216			
α-Tocopherol	Mean	4.76	4.82			
	Range	<0.500[b] - 9.41	<0.500[b] - 10.8			
	Confidence Interval	2.49 - 7.02	2.55 - 7.08	0 - 22.5	NQ - 68.67	<0.500[b] - 28.2
	Adjusted P-Value	--	0.955			
	P-Value	--	0.761			
	Mean	0.273	0.273	<0.500[b] - 1.10[r]	NQ - 19.80	<0.500[b] - 1.85
	Range	<0.500[b] - 0.981	<0.500[b] - 0.992			

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
β-Tocopherol	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
γ-Tocopherol	Mean	13.2	13.2	0 - 43.8	NQ - 58.61	1.49 - 47.6
	Range	2.19 - 22.5	2.12 - 22.2			
	Confidence Interval	7.68 - 18.7	7.70 - 18.8			
	Adjusted P-Value	--	0.981			
	P-Value	--	0.968			
δ-Tocopherol	Mean	0.400	0.417	<0.500[b] - 1.81	NQ - 14.61	<0.500[b] - 2.53
	Range	<0.500[b] - 1.18	<0.500[b] - 1.75			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Total Tocopherols	Mean	18.6	18.8	0 - 57.1	NQ - 89.91	2.24 - 61.9
	Range	2.94 - 31.4	3.20 - 32.7			
	Confidence Interval	10.8 - 26.3	11.1 - 26.5			
	Adjusted P-Value	--	0.871			
	P-Value	--	0.589			

Note: This table provides results from the mixed model analysis only. Not applicable (NA); mixed model analysis was not performed, or confidence interval was not determined. Not quantified (NQ); one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified. Vitamin composition is reported as mg/kg Dry Weight.

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

[r] 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.

Secondary Metabolites and Anti-Nutrients Assessment of DP51291 Maize Grain

Secondary metabolite and anti-nutrients were analyzed in grain derived from DP51291 maize and control maize. Results are shown in Table 31. No statistically significant differences (P-value < 0.05) were observed between DP51291 maize and control maize, with an exception for one analyte. A statistically significant difference (P-value <0.05) was observed between DP51291 maize and control maize for trypsin inhibitor. All individual values for this analyte were within the tolerance interval, indicating DP51291 maize is within the range of biological variation for this analyte and the statistical difference is not biologically meaningful. The non-significant FDR-adjusted P-value for trypsin inhibitor indicates that this difference was likely a false positive.

These results demonstrate that the secondary metabolite and anti-nutrient composition of grain derived from DP51291 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 31. Secondary Metabolite and Anti-Nutrient Results for DP51291 Maize Grain

Analyte	Reported Statistics	Control Maize	Herbicide-Treated DP51291 Maize	Tolerance Interval	Literature Range	Reference Data Range
p-Coumaric Acid	Mean	0.0210	0.0206	0.00786 - 0.0478	NQ - 0.08	0.0134 - 0.0469
	Range	0.0159 - 0.0263	0.0150 - 0.0282			
	Confidence Interval	0.0193 - 0.0227	0.0189 - 0.0223			
	Adjusted P-Value	--	0.834			
	P-Value	--	0.500			
Ferulic Acid	Mean	0.245	0.237	0.0822 - 0.329	0.02 - 0.44	0.167 - 0.334
	Range	0.176 - 0.294	0.188 - 0.289			
	Confidence Interval	0.230 - 0.259	0.222 - 0.251			
	Adjusted P-Value	--	0.341			
	P-Value	--	0.0501			
Furfural	Mean	<0.000100[b]	<0.000100[b]	<0.0000500[b]	NQ	<0.000100[b]
	Range	<0.000100[b]	<0.000100[b]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Inositol	Mean	0.0228	0.0234	0.00959 - 0.0567	0.00613 - 0.257	0.0102 - 0.0604
	Range	0.0117 - 0.0415	0.0104 - 0.0386			
	Confidence Interval	0.0171 - 0.0284	0.0178 - 0.0291			
	Adjusted P-Value	--	0.834			
	P-Value	--	0.457			
Phytic Acid	Mean	1.02	0.990	0.511 - 1.31	NQ - 1.940	0.597 - 1.15
	Range	0.725 - 1.22	0.689 - 1.26			
	Confidence Interval	0.940 - 1.10	0.912 - 1.07			
	Adjusted P-Value	--	0.637			
	P-Value	--	0.225			
Raffinose	Mean	0.102	0.112	0 - 0.393	NQ - 0.466	<0.0800[b] - 0.364
	Range	<0.0800[b] - 0.242	<0.0800[b] - 0.291			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Trypsin Inhibitor (TIU/mg DW)	Mean	1.39	1.27	0.170 - 5.65	NQ - 8.42	0.491 - 2.45
	Range	0.563 - 2.47	0.615 - 2.04			
	Confidence Interval	1.21 - 1.57	1.08 - 1.45			
	Adjusted P-Value	--	0.271			
	P-Value	--	0.0319*			

Note: This table provides results from the mixed model analysis only. Not applicable (NA); mixed model analysis was not performed, or confidence interval was not determined. Not quantified (NQ); one or more assay values in the published literature references were below the lower limit of quantification (LLOQ) and were not quantified. Secondary Metabolite and Anti-Nutrient Composition (% Dry Weight or as Indicated).

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

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

Conclusions on the Food and Feed Safety Assessment of DP51291 Maize

The compositional equivalence of DP51291 maize to a conventional non-GM comparator with a history of safe use in food and feed was assessed. The results demonstrated that nutrient composition of forage and grain derived

from DP51291 maize is comparable to that of conventional maize represented by non-GM near-isoline maize and non-GM commercial maize. Based on these analyses, the grain and forage of DP51291 maize are comparable to that of conventional maize with respect to nutrient composition.

C INFORMATION RELATED TO THE NUTRITIONAL IMPACT

As seen in above Section *B.5 Compositional analyses* did not indicate any biologically relevant changes to the levels of nutrients in the forage and grain derived from DP51291 maize compared to the non-GM counterpart. The results demonstrated that nutrient composition of forage and grain derived from DP51291 maize was comparable to that of conventional maize represented by non-GM near-isoline maize and non-GM commercial maize.

D OTHER INFORMATION

Overall risk assessment conclusions for DP51291 maize

This application presents information supporting the safety and nutritional comparability of DP51291 maize. The molecular characterization analyses conducted on DP51291 maize demonstrated that the introduced genes are integrated at a single locus, stably inherited across multiple generations, and segregated according to Mendel's law of genetics. The toxicity and allergenicity potential of the IPD072Aa, PAT, and PMI proteins were evaluated and found unlikely to be toxic or allergenic to humans or animals. Based on the weight of evidence, consumption of the IPD072Aa, PAT, and PMI proteins is unlikely to cause an adverse effect on humans or animals. A compositional equivalence assessment demonstrated that the nutrient composition of DP51291 maize forage and grain is comparable to that of conventional maize, represented by non-genetically modified (non-GM) near-isoline maize and non-GM commercial maize.

Overall, data and information contained herein support the conclusion that DP51291 maize containing the IPD072Aa, PAT, and PMI proteins is as safe and nutritious as non-GM maize for food and feed uses.

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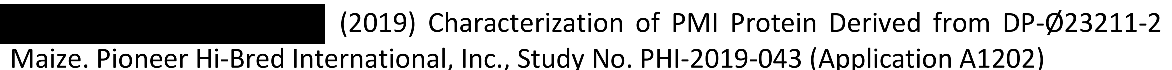 (2019) Characterization of PMI Protein Derived from DP-Ø23211-2 Maize. Pioneer Hi-Bred International, Inc., Study No. PHI-2019-043 (Application A1202)

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APPENDIX A. METHODS FOR SOUTHERN-BY-SEQUENCING ANALYSIS ([redacted], 2022 (PHI-2022-120 STUDY))

Test, Control and Reference Substances

The test substance in this study was defined as the DP-Ø51291-2 event contained within seed from the segregating T1 generation of DP51291 maize. The control substance was defined as the absence of the DP-Ø51291-2 event in untransformed PHR03 maize seed (referred to as control maize). The unmodified line has a genetic background representative of the test substance background; however, it does not contain the DP51291 insertion.

DNA Extraction and Quantitation

Genomic DNA was extracted from leaf tissue of DP51291 and control maize plants. DNA was extracted using the sbeadex maxi plant kit (LGC Genomics) and the Kingfisher Flex instrument (Thermo Scientific). Following extraction, the extracted DNA was assessed using a DropSense96 (Unchained Labs) and DNA was stored at 4 °C.

Southern-by-Sequencing

SbS was performed by Pioneer Hi-Bred International, Inc. Genomics Technologies. SbS analysis utilizes probe-based sequence capture, Next Generation Sequencing (NGS) techniques, and bioinformatics procedures to capture, sequence, and identify inserted DNA within the maize genome (Zastrow-Hayes et al., 2015). By compiling a large number of unique sequencing reads and mapping them against the intended insertion sequence (comprising the intended expression cassettes from [redacted] and the landing pad sequences from [redacted] including the complete [redacted] and [redacted] elements), linearized transformation plasmid maps, and the endogenous genomic reference, unique junctions due to inserted DNA are identified in the bioinformatics analysis. This information is used to determine the number and organization of insertions within the plant genome and confirm the absence of plasmid backbone or other unintended plasmid sequences.

Genomic DNA samples isolated from ten individual plants of the T1 generation of DP51291 maize (five transgenic plants and five null segregant plants) were analyzed by SbS to determine the insertion copy number and organization and to confirm the absence of plasmid backbone or other unintended plasmid sequences. SbS was also performed on control maize DNA and positive control samples (control maize DNA spiked with [redacted] plasmid DNA) to confirm that the assay could reliably detect plasmid fragments within the genomic DNA.

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

Capture Probe Design and Synthesis

Biotinylated capture probes used to select plasmid sequences were designed and synthesized by Roche NimbleGen, Inc. The probe set was designed to target all sequences the [redacted] plasmids.

Sequencing Library Construction

NGS libraries were constructed for DNA samples from individual maize plants, including plants from the T1 generation of DP51291 maize, a control maize plant, and the positive control samples. Genomic DNA isolated 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 Unique Dual Index barcode adaptors (Bioo Scientific Corp.) following the kit protocol so that samples would be indexed to enable identification after sequencing. The DNA fragment libraries were amplified by PCR for eight cycles prior to the capture process. Amplified libraries were analyzed using a DropSense96, diluted to 5 ng/µl with nuclease-free water.

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.

Next Generation Sequencing on Illumina Platform

Following sequence capture, the libraries were submitted for NGS (Illumina NextSeq2000) to targeted depth of approximately 100x for the captured sequences. The sequence reads were trimmed [redacted] (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.

Due to low read counts in the initial sequencing run, a second sequencing run was performed with the same final double-enriched library pool and the reads from the two sequencing runs were combined for the analysis of each sample.

Quality Assurance of Sequencing Reads

The adapter sequences were trimmed from the NGS sequence reads using Cutadapt, v2.10 (Martin, 2011). Further analysis to eliminate sequencing errors used JELLYFISH, version 2.2.10 (Marçais and Kingsford, 2011), [redacted] 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 (referred to as "Non-redundantReads") while retaining abundance information for each group and were used for further analysis, as described in Zastrow-Hayes et al. (2015).

Aligning Reads

Each set of "Non-redundantReads" was aligned to the plasmid sequences, including the plasmid backbone sequences, using Bowtie2, version 2.3.4.2 (Langmead and Salzberg, 2012), [redacted]. Remaining "Non-redundantReads" were aligned to the maize reference genome using Bowtie2, version 2.3.4.2, [redacted]

Junction Detection

Following removal of "Non-redundantReads" with alignments to the endogenous maize reference or plasmid sequence identified during the quality assurance phase, the remaining "Non-redundantReads" were aligned to the

full plasmid sequences using Bowtie2, version 2.3.4.2, with the soft-trimming feature enabled. Chimeric reads contain sequence that is non-contiguous with the plasmid sequence from the alignment, such as genome-plasmid junctions or rearrangements of the plasmid. These chimeric reads are referred to as junction reads or junctions. This identifier (referred to as a 30_20-mer) includes 20 bp of sequence from , and 30 bp of sequence adjacent to the plasmid-derived 20 bp within a sequencing read. The adjacent 30 bp would either align to the genome or to part of the plasmid sequence that was not contiguous to the known 20 bp. When the 20 bp from the plasmid and the adjacent 30 bp were identified as a 30_20-mer, they indicated the junction shown by the chimeric read. The total number of sequencing reads (referred to as "TotalSupportingReads") for each junction was retained for filtering.

Junction Identification

Variations between the endogenous genomic reference used in the SbS analysis and the control maize genome may result in identification of junctions that are due to these differences. In order to detect these endogenous junctions, control maize genomic DNA library was captured and sequenced in the same manner. The 30_20-mers of the endogenous junctions detected in the control sample were used to filter the same endogenous junctions in the DP51291 maize samples, so that the only junctions remaining in the DP51291 samples are due to insertions derived from .

Data QC

The transgenic and null samples were compared to the control maize sample and a quality check was performed. If regions of the plasmid backbone or other unintended sequences contain low to medium sequencing coverage compared to the control maize sample and no junctions were identified, the data was reviewed a second time. If the review confirmed the absence of junctions, this indicates that no plasmid sequence was inserted into the genome. Contamination is a possible source of such reads and the sequencing reads will be reviewed to determine the type of contaminant. If the sample is contaminated by a different sample that contains a similar plasmid with overlapping elements, most if not all of the sequencing reads will align to the plasmid being analyzed. If the sample is contaminated from a bacterial source that lives on the plant, the sequencing reads will likely not align exactly to the plasmid.

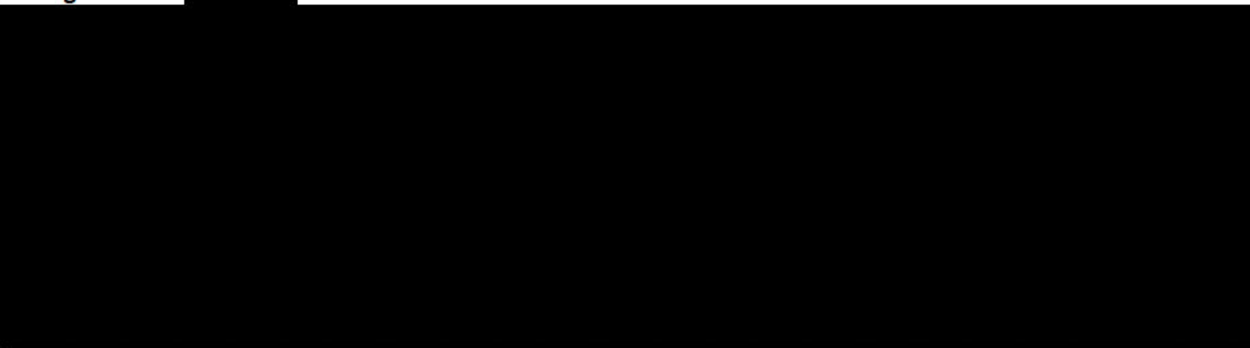
SbS Results

Results for the control maize, positive control, and one DP51291 maize plant (Plant ID 434578663) null segregant (negative) plant (Plant ID 434578664) are presented in the main body (Section *A.3(c) Molecular characterisation*) of this document. Remaining plant results from SbS analysis are presented in Figures A1 to A4 (positive plants) below.

A. Alignment to Intended Insertion

B. Alignment to

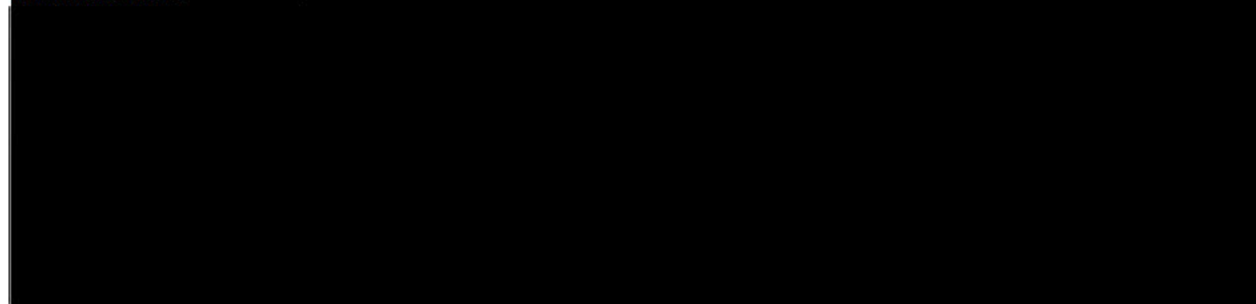
C. Alignment to



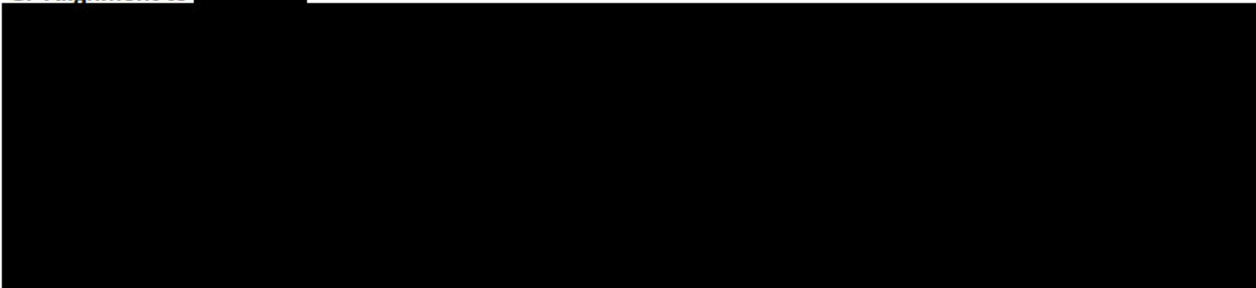
D. Alignment to

E. Alignment to

F. Alignment to



G. Alignment to



H. Alignment to

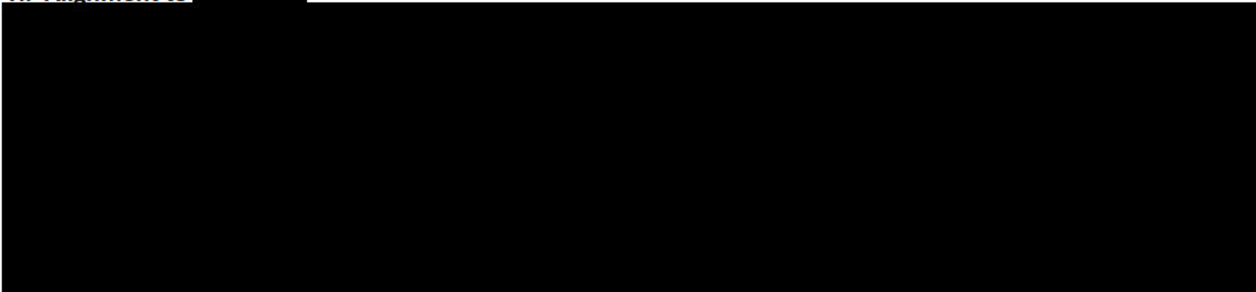


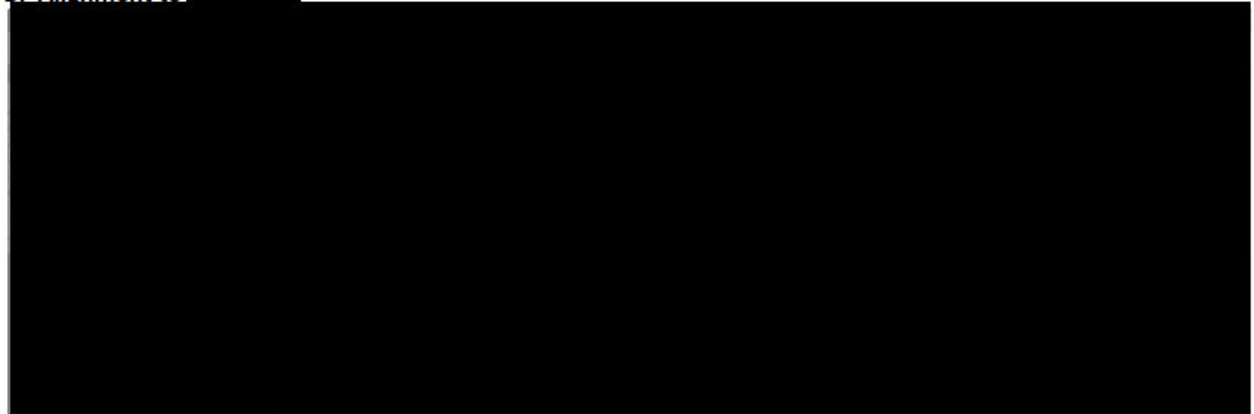
Figure A1. SbS Results for Plant ID 434578667 – DP51291 Maize (Transgenic)

The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion (bp; Figure 11), indicating that this plant contains the intended insertion. The bottom part of the graph shows the two plasmid-to-genome sequence junctions identified by SbS; the numbers refer to the bp location of each junction relative to the intended insertion (Figure 11). The presence of only two junctions demonstrates the presence of a single insertion in the DP51291 maize genome. **B)** SbS results aligned against the plasmid sequence bp; Figure 9). Coverage was obtained for the elements between transferred into DP51291 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region between the RB and FRT1 that were not transferred into the DP51291 maize genome, and to the terminator (*), terminator (†), and site (‡) elements outside of the FRT sites, due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in . Finally, coverage was obtained to the regions around the RB

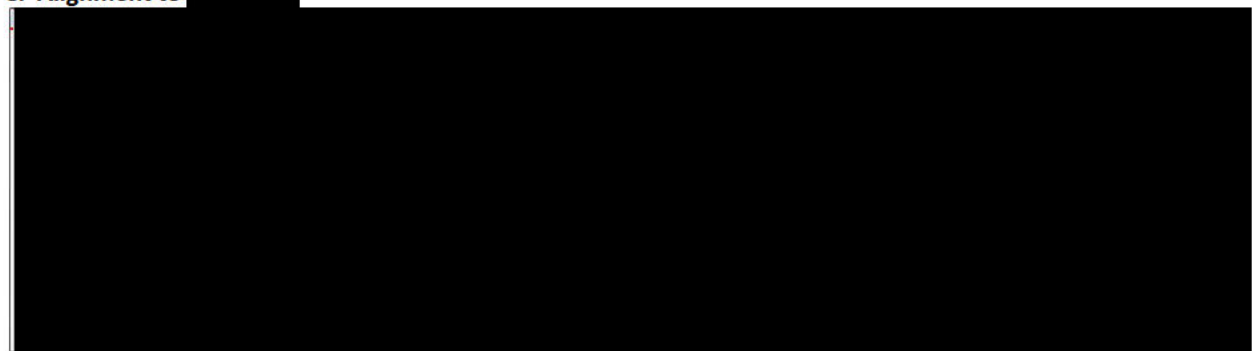
and LB elements that match the corresponding sequences derived from **C)** SbS results aligned against the plasmid sequence bp; Figure 3). Coverage was obtained for the elements found in the intended insertion (between the to the first site, the second to and between to the), along with the endogenous intron element, an site (‡) and the cassettes between the FRT sites that are identical to those found in the final DP51291 maize insertion. **D)** SbS results aligned against the plasmid sequence bp; Figure 5). Coverage was obtained only for the endogenous elements along with the terminator element (*). **E)** SbS results aligned against the plasmid sequence bp; Figure 4). Coverage was obtained only for the endogenous elements along with the terminator element (*). **F)** SbS results aligned against the plasmid sequence bp; Figure 3). Coverage was obtained for the endogenous elements along with the FRT sites, terminator elements (*), terminator (†), and sites (‡) that are identical to sequences in the DP51291 maize insertion. **G)** SbS results aligned against the plasmid sequence (bp; Figure 6). Coverage was obtained for the endogenous elements. The coverage at approximately bp is due to a bp region that is an exact match to a region in the insertion derived from **H)** SbS results aligned against the plasmid sequence bp; Figure 7). Coverage was obtained for the endogenous elements along a bp region that is identical to the element in the insertion derived from (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or plasmid backbone or other unintended sequences present in DP51291 maize.

A. Alignment to Intended Insertion

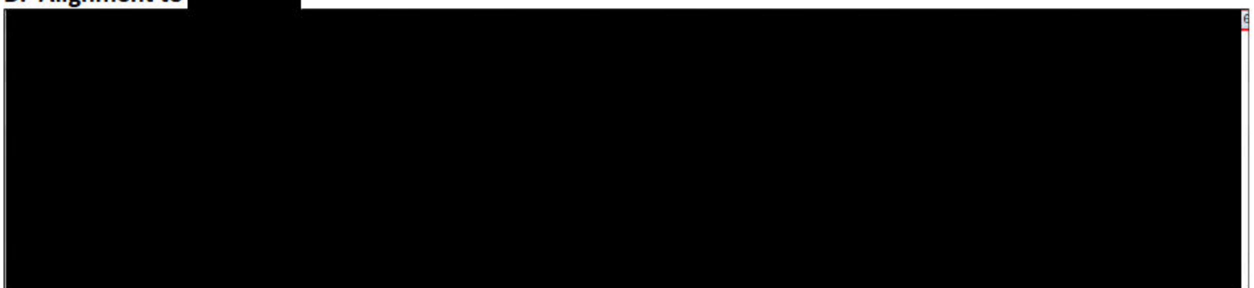
B. Alignment to



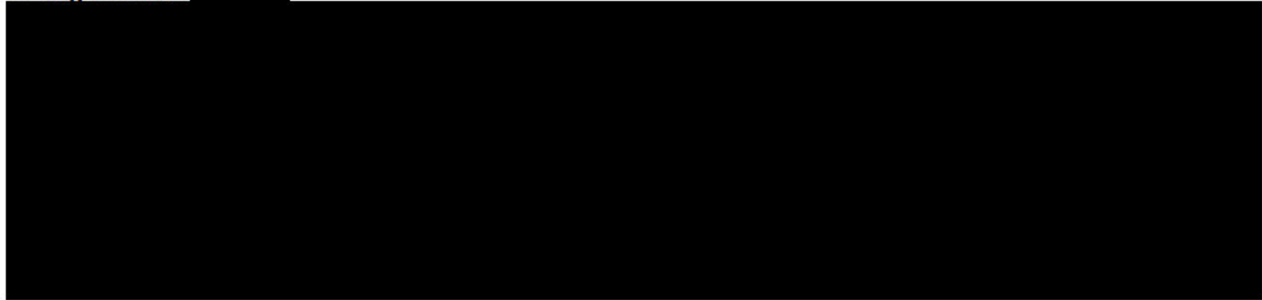
C. Alignment to



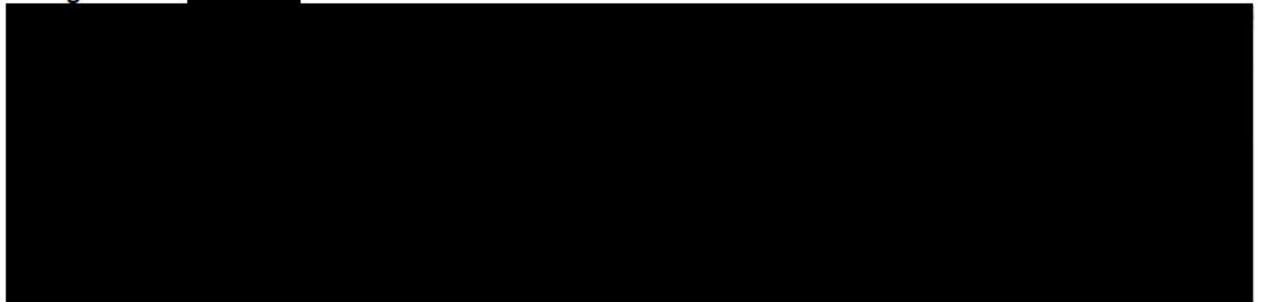
D. Alignment to



E. Alignment to



F. Alignment to



G. Alignment to

H. Alignment to

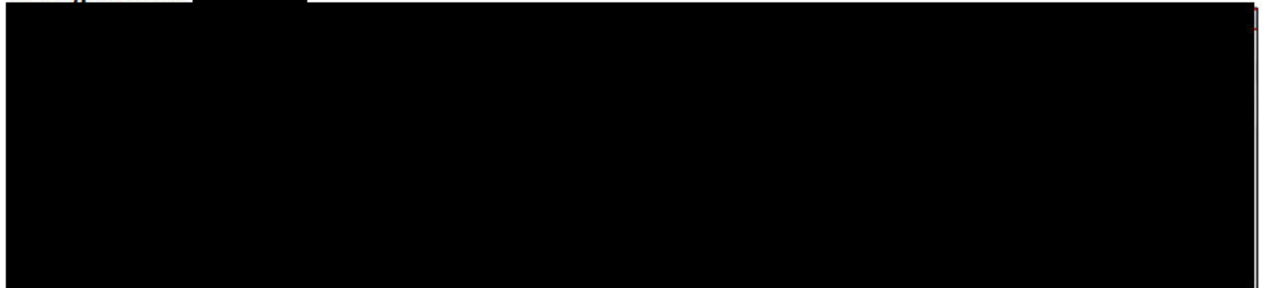
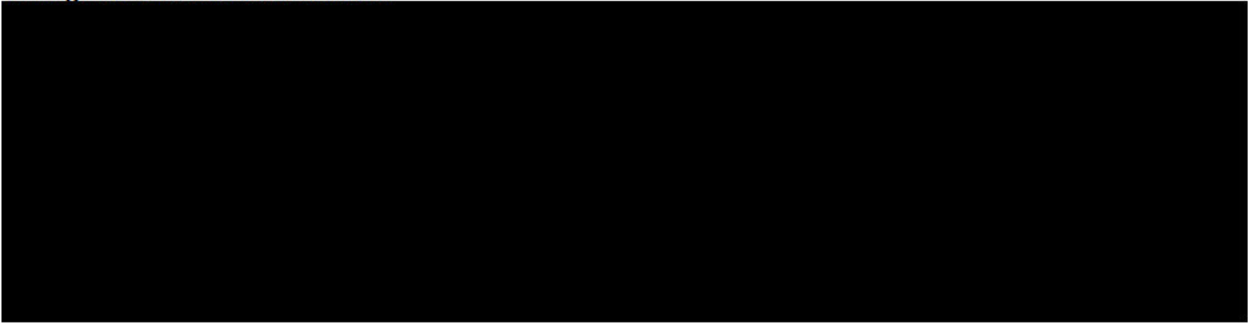


Figure A2. SbS Results for Plant ID 434578672 – DP51291 Maize (Transgenic)

The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion bp; Figure 11), indicating that this plant contains the intended insertion. The bottom part of the graph shows the two plasmid-to-genome sequence junctions identified by SbS; the numbers refer to the bp location of each junction relative to the intended insertion (Figure 11). The presence of only two junctions demonstrates the presence of a single insertion in the DP51291 maize genome. **B)** SbS results aligned against the plasmid sequence bp; Figure 9). Coverage was obtained for the elements between transferred into DP51291 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region between the that were not transferred into the DP51291 maize genome, and to the terminator (*), terminator (†), and site (‡) elements outside of the FRT sites, due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in . Finally, coverage was obtained to the regions around the RB and LB elements that match the corresponding sequences derived from **C)** SbS results aligned against the plasmid sequence

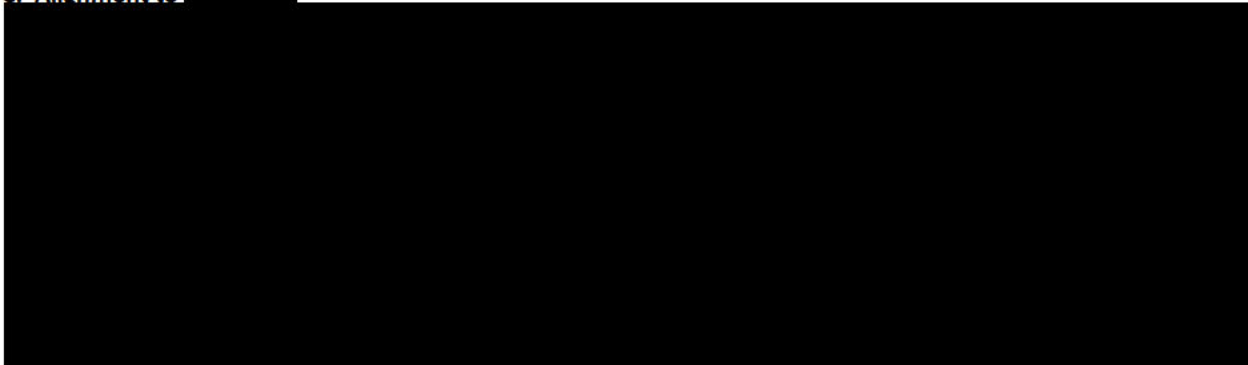
bp; Figure 1). Coverage was obtained for the elements found in the intended insertion (between the to the first site, the second to and between to the), along with the endogenous intron element, an site (‡) and the cassettes between the FRT sites that are identical to those found in the final DP51291 maize insertion. **D)** SbS results aligned against the plasmid sequence bp; Figure 5). Coverage was obtained only for the endogenous elements along with the terminator element (*). **E)** SbS results aligned against the plasmid sequence bp; Figure 4). Coverage was obtained only for the endogenous elements along with terminator element (*). **F)** SbS results aligned against the plasmid sequence bp; Figure 3). Coverage was obtained for the endogenous elements along with the FRT sites, terminator elements (*), terminator (†), and sites (‡) that are identical to sequences in the DP51291 maize insertion. **G)** SbS results aligned against the plasmid sequence (bp; Figure 6). Coverage was obtained for the endogenous elements. The coverage at approximately bp is due to a -bp region that is an exact match to a region in the insertion derived from . **H)** SbS results aligned against the plasmid sequence (bp; Figure 7). Coverage was obtained for the endogenous elements along a -bp region that is identical to the element in the insertion derived from (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or plasmid backbone or other unintended sequences present in DP51291 maize.

A. Alignment to Intended Insertion

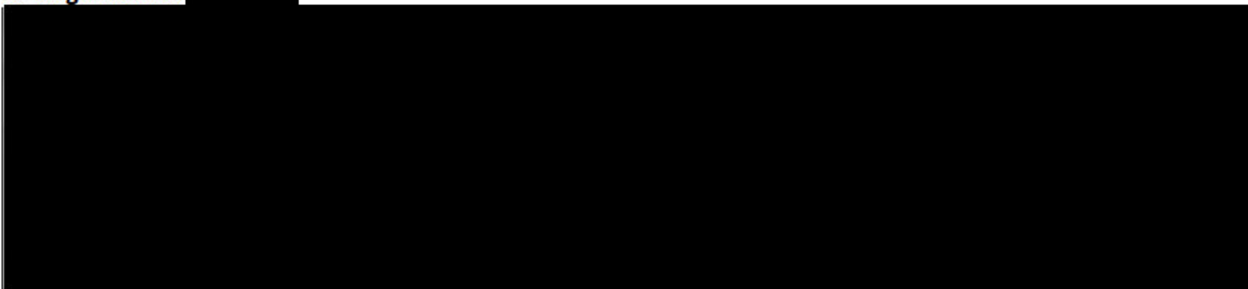


B. Alignment to

C. Alignment to



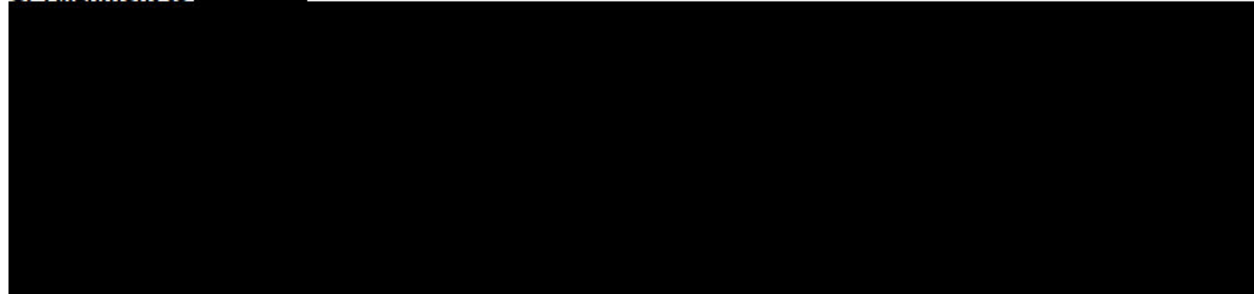
D. Alignment to



E. Alignment to

F. Alignment to

G. Alignment to



H. Alignment to

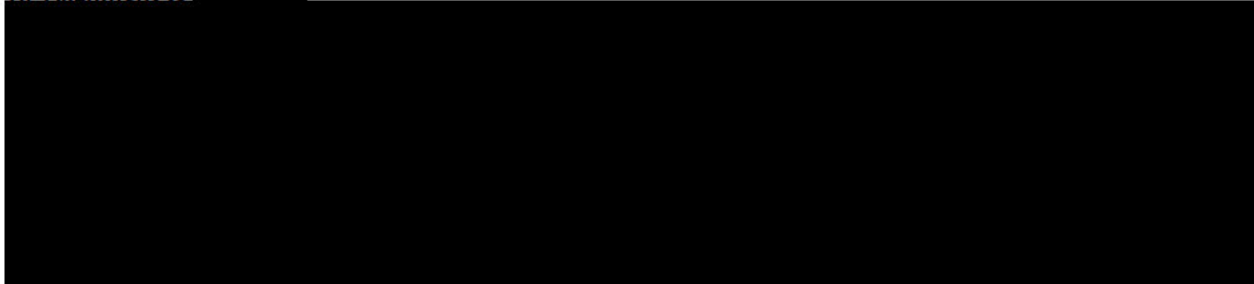


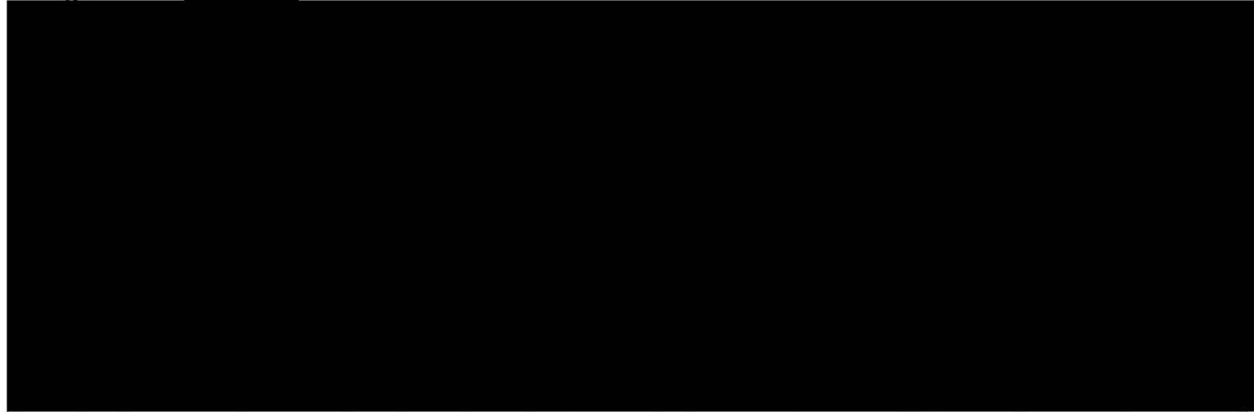
Figure A3. SbS Results for Plant ID 434578673 – DP51291 Maize (Transgenic)

The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion bp; Figure 11), indicating that this plant contains the intended insertion. The bottom part of the graph shows the two plasmid-to-genome sequence junctions identified by SbS; the numbers refer to the bp location of each junction relative to the intended insertion (Figure 11). The presence of only two junctions demonstrates the presence of a single insertion in the DP51291 maize genome. **B)** SbS results aligned against the plasmid sequence bp; Figure 9). Coverage was obtained for the elements between transferred into DP51291 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region between the that were not transferred into the DP51291 maize genome, and to the terminator (*), terminator (†), and site (‡) elements outside of the FRT sites, due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in Finally, coverage was obtained to the regions around the RB and LB elements that match the corresponding sequences derived from **C)** SbS results aligned against the plasmid sequence

bp; Figure 1). Coverage was obtained for the elements found in the intended insertion (between the to the first site, the second to and between to the), along with the endogenous intron element, an site (‡) and the cassettes between the FRT sites that are identical to those found in the final DP51291 maize insertion. **D)** SbS results aligned against the plasmid sequence bp; Figure 5). Coverage was obtained only for the endogenous elements along with the terminator element (*). **E)** SbS results aligned against the plasmid sequence bp; Figure 4). Coverage was obtained only for the endogenous elements along with the terminator element (*). **F)** SbS results aligned against the plasmid sequence bp; Figure 3). Coverage was obtained for the endogenous elements along with the FRT sites, terminator elements (*), terminator (†), and sites (‡) that are identical to sequences in the DP51291 maize insertion. **G)** SbS results aligned against the plasmid sequence bp; Figure 6). Coverage was obtained for the endogenous elements. The coverage at approximately bp is due to a -bp region that is an exact match to a region in the insertion derived from . **H)** SbS results aligned against the plasmid sequence bp; Figure 7). Coverage was obtained for the endogenous elements along a -bp region that is identical to the element in the insertion derived from (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or plasmid backbone or other unintended sequences present in DP51291 maize.

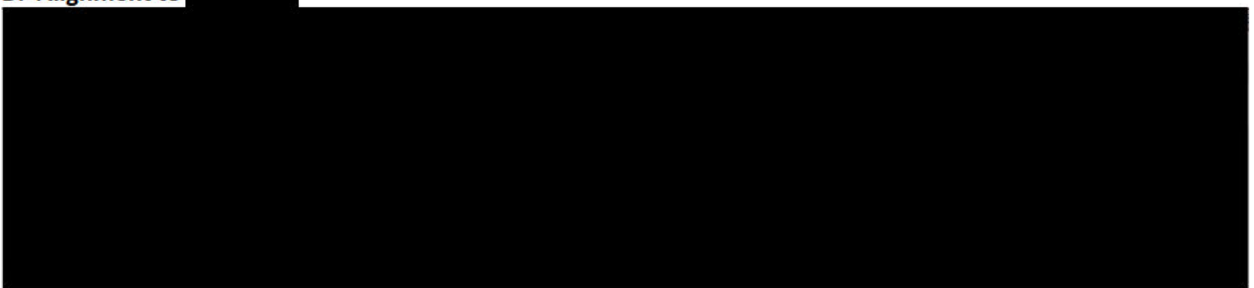
A. Alignment to Intended Insertion

B. Alignment to



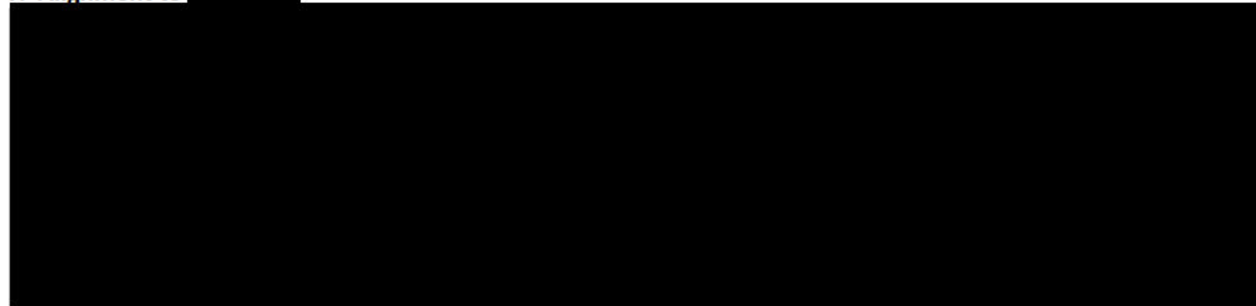
C. Alignment to

D. Alignment to

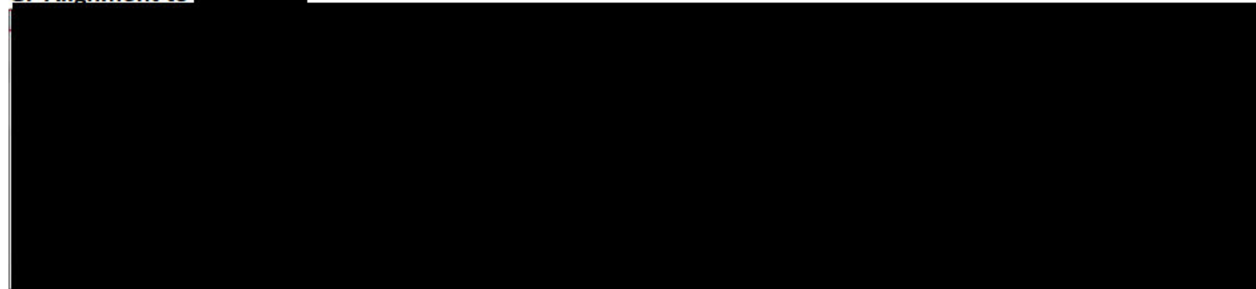


E. Alignment to

F. Alignment to



G. Alignment to



H. Alignment to

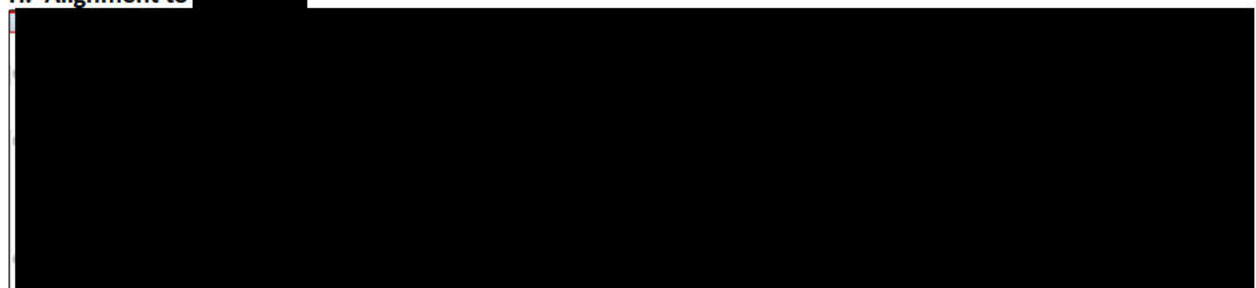


Figure A4. SbS Results for Plant ID 434578675 – DP51291 Maize (Transgenic)

The blue coverage graph shows the number of individual NGS reads aligned at each point on the intended insertion or plasmid using a logarithmic scale at the middle of the graph. Green bars above the coverage graph indicate endogenous genetic elements in each plasmid derived from the maize genome (identified by numbers; Table 6), while tan bars indicate genetic elements derived from other sources. FRT sites are indicated by red arrows. **A)** SbS results aligned against the intended insertion bp; Figure 11), indicating that this plant contains the intended insertion. The bottom part of the graph shows the two plasmid-to-genome sequence junctions identified by SbS; the numbers refer to the bp location of each junction relative to the intended insertion (Figure 11). The presence of only two junctions demonstrates the presence of a single insertion in the DP51291 maize genome. **B)** SbS results aligned against the plasmid sequence bp; Figure 9). Coverage was obtained for the elements between transferred into DP51291 maize (region between the red arrows at top of graph). Coverage was also obtained for the endogenous elements in the region between the that were not transferred into the DP51291 maize genome, and to the terminator (*), terminator (†), and site (‡) elements outside of the FRT sites, due to alignment of reads derived from identical elements in the final insertion to all copies of these elements in Finally, coverage was obtained to the regions around the RB

and LB elements that match the corresponding sequences derived from [redacted] **C)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 1). Coverage was obtained for the elements found in the intended insertion (between the [redacted] to the first [redacted] site, the second [redacted] to [redacted] and between [redacted] to the [redacted]), along with the endogenous [redacted] intron element, an [redacted] site (‡) and the cassettes between the FRT sites that are identical to those found in the final DP51291 maize insertion. **D)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 5). Coverage was obtained only for the endogenous elements along with the [redacted] terminator element (*). **E)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 4). Coverage was obtained only for the endogenous elements along with the [redacted] terminator element (*). **F)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 3). Coverage was obtained for the endogenous elements along with the FRT sites, [redacted] terminator elements (*), [redacted] terminator (†), and [redacted] sites (‡) that are identical to sequences in the DP51291 maize insertion. **G)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 6). Coverage was obtained for the endogenous elements. The coverage at approximately [redacted] bp is due to a [redacted] bp region that is an exact match to a region in the insertion derived from [redacted]. **H)** SbS results aligned against the plasmid [redacted] sequence [redacted] bp; Figure 7). Coverage was obtained for the endogenous elements along a [redacted] bp region that is identical to the [redacted] element in the insertion derived from [redacted] (‡). The absence of any junctions other than to the intended insertion indicates that there are no additional insertions or plasmid backbone or other unintended sequences present in DP51291 maize.

APPENDIX B. METHODS FOR SOUTHERN BLOT ANALYSIS ([redacted], 2022 (PHI-2022-064 STUDY))

Test, Control and Reference Substances

The test substances in the study were defined as DP-Ø51291-2 contained within seeds from the T1, T2, T3, T4, and T5 generations of DP51291 maize. The control substance was defined as seed from a maize line (PHR03) that was not transformed. PHR03 maize has a similar genetic background to the test substance; however, it does not contain the DP51291 maize insertion.

Plasmid DNA of [redacted] that was used for transformation to produce DP51291 maize was defined as a reference substance. This plasmid was used as a positive control for Southern analysis to verify probe hybridization. The *pmi, mo-pat* and *ipd072Aa* probes used in this analysis were derived from plasmid [redacted].

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

Sample Collection, Handling, Identification and Storage

Seed from each of the five generations of DP51291 maize and the control maize were planted in a controlled environment at Pioneer (Johnston, Iowa, USA). Fresh leaf tissue samples from test and control maize were harvested, stored frozen (≤ -50 ºC freezer unit), and then lyophilized. Lyophilized tissue samples were shipped to Regulatory Sciences, Multi Crop Research Center, Pioneer Hi-Bred Private Limited at Hyderabad, at ambient temperature. Upon arrival, samples were stored frozen (≤ -50 °C freezer unit) until processing.

DNA Extraction and Quantification

Genomic DNA was isolated and analyzed from one plant for each of the T1, T2, T3, T4, and T5 generations of DP51291 maize and one plant from the PHR03 control maize.

The lyophilized leaf samples were pulverized with steel beads in tubes using a paint shaker (AGS Transact Technology Ltd.,). Care was taken to ensure leaf samples were ground sufficiently for DNA isolation. Genomic DNA was isolated using a high salt extraction buffer (2.0 M Sodium chloride, 100 mM Tris-Hydrochloride pH-8.0, 50 mM Sodium salt of EDTA, 3% β-mercaptoethanol (v/v) and 100 mM Sodium metabisulphite) and sequentially precipitated using potassium acetate and isopropyl alcohol. Extracted DNA was treated with Ribonuclease A (RNase A), purified using phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated using sodium acetate and chilled ethanol. Following the extraction, DNA was quantified using Quant-iT™ PicoGreen® reagent (Molecular Probes, Invitrogen) and visualized on a 1% agarose gel to check the quality of the isolated DNA.

Digestion of DNA and Electrophoretic Separation

Genomic DNA isolated from both test and control maize leaves was digested with the restriction enzyme [redacted] (Thermo Fisher Scientific.). Plasmid DNA was added to the control maize DNA samples at a level equivalent to one

plasmid copy per genomic copy and digested in the same manner. Following digestion with the restriction enzyme, the fragments produced were electrophoretically separated according to their sizes on a 0.9% agarose gel. After electrophoresis, the gel was stained using GelRed (Biotium Inc.) and documented by photographing the gel under UV illumination (BioRad Gel doc XR^+ System).

Southern Transfer

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

Probe Labeling and Southern Blot Hybridization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probe. DNA probes specific to the *pmi, mo-pat* and *ipd072Aa* gene elements were labeled by incorporation of Digoxigenin (DIG) labeled nucleotide DIG-11-dUTP into the fragments.

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

Detection of Hybridized Probes

After overnight hybridization, the membrane was washed and processed using the DIG Wash and Block Buffer Set (Roche). DIG-labeled DNA standards and single stranded DIG-labeled probes hybridized to DNA bound to the nylon membrane were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System provided in DIG Wash and Block Buffer Set (Roche). Blots were exposed for one or more time points to detect hybridizing fragments and to visualize molecular weight standards. Images were captured by detection with the Syngene G-Box Chemi XX6 (Syngene, Inc.). Detected bands were documented for each probe.

Stripping of Probes and Subsequent Hybridization

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

APPENDIX C. METHODS FOR MULTI-GENERATION SEGREGATION ANALYSIS ([redacted] ET AL., 2022 (PHI-2018-035 STUDY))

Five generations of DP51291 maize were evaluated using polymerase chain reaction (PCR) analyses and herbicide-tolerance testing to confirm Mendelian inheritance of genotype and phenotype.

Greenhouse Experimental Design

Five separate generations (T1, T2, T3, T4, and T5) of DP51291 maize were planted and grown in a greenhouse under standard environmental conditions for maize production. Leaf punch samples were collected from all five generations for real-time quantitative polymerase chain reaction (qPCR) and qualitative polymerase chain reaction (PCR) analysis. The genotypic analysis evaluated each individual plant for the copy number of the [redacted] insertion site representing event DP-Ø51291-2 and the *ipd072Aa, mo-pat,* and *pmi* genes as well as for the presence or absence of specific genetic elements. After sample collection, the plants were treated with a broadcast application of glufosinate and then visually evaluated for herbicide tolerance. The individual results for each plant were compared to the qPCR and PCR results to verify co-segregation of genotype and phenotype.

Planting and Leaf Sample Collection

Maize seeds, one hundred sixty-five seeds for each generation, were planted in separate pots intended for genotypic and phenotypic analysis. All seeds were grown in a controlled environment under suitable conditions for producing maize plants. After germination but prior to qPCR and PCR sample collection, each generation was thinned by removing unhealthy plants. All non-selected plants were removed and discarded.

Leaf punch samples were collected from each plant in all entries of the T1, T2, T3, T4, and T5 generations of DP51291 maize as well as the control maize. Each sample consisted of three leaf punches collected into one bullet tube and placed on dry ice until transferred to a freezer. Individual plant and corresponding leaf samples were uniquely labeled to allow a given sample to be tracked back to the originating plant.

Genotypic Analysis

Following DNA extraction, the leaf punch samples were analyzed using real-time qPCR or PCR to determine the copy number of the [redacted] insertion site representing event DP-Ø51291-2 and the *ipd072Aa, mo-pat,* and *pmi* genes as well as qualitative PCR analysis (PCR) to determine the presence or absence of specific genetic elements.

Phenotypic Analysis

Following leaf punch sampling, 1X glufosinate was applied to the maize plants at approximately the V3 or V4 growth stage (occurs when the collar of the third or fourth leaf becomes visible, respectively). The spray mixture consisted of Ignite 280 SL containing 2.34 pounds active ingredient of glufosinate per gallon (280 g ai/L) 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.

Four to five days after herbicide application, each plant was visually evaluated for herbicide tolerance in which presence of herbicide injury corresponded to an herbicide-susceptible phenotype and absence of herbicide injury corresponded to an herbicide-tolerant phenotype.

Statistical Analysis

A chi-square analysis was performed at the 0.05 significance level on the segregation results of each DP51291 maize generation to compare the observed segregation ratio to the expected segregation ratio (1:1 for T1 generation, 3:1 for T2 and T3 generations). 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 the T4 and T5 generations because all plants were identified as positive (*i.e.*, not segregating) as expected for a homozygous generation.

APPENDIX D. METHODS FOR CHARACTERISATION OF IPD072AA PROTEIN (ET AL., 2022 (PHI-2022-054 STUDY))

Test Materials

Maize-Derived IPD072Aa Protein: IPD072Aa protein was isolated from DP51291 maize whole plant tissue. The tissue samples were collected at the V9 growth stage (the stage when the collar of the ninth leaf becomes visible; Abendroth et al., 2011) of development from plants grown at a field location in Johnston, IA, USA. The tissue was lyophilized, homogenized and stored at ≤ -50 °C.

The IPD072Aa protein was extracted from lyophilized maize tissue by homogenization with a pre-chilled Waring blender using phosphate-buffered saline containing polysorbate 20 (PBST) with 5% (w/v) non-fat dry milk extraction buffer. The sample extract was then clarified by filtration and centrifugation. Ammonium sulfate (AS) precipitation was used to further concentrate the sample extract. Beginning at 0% AS saturation, AS was slowly added to the sample extract while stirring until 60% AS saturation was reached. The sample was centrifuged and the AS process was repeated with the supernatant, this time beginning at 60% AS saturation and progressing to 80%. The sample was centrifuged again, and the fractionated pellets were stored frozen (-80 °C freezer unit). The fractionated pellet of the sample was solubilized in phosphate-buffered saline prior to running the sample through a Econo Pac 10DG desalting column (BioRad). After desalting, the eluted fraction was further purified by immunoaffinity chromatography. The immunoaffinity column was prepared by coupling an IPD072Aa protein mouse monoclonal antibody (21F1.E5) to AminoLink Plus Coupling Resin. Elutions 2-5 from the immunoaffinity purification were concentrated into one sample using a centrifugal concentrator (10K Vivaspin; Sartorius). Following extraction, purification, and concentration, the final volume in the concentrator was estimated and 25% 4X NuPAGE LDS and 10% 10X NuPAGE Sample Reducing Agent was added to the concentrated sample. The sample in the concentrator was heated for 2-5 minutes at 70-100 °C and then transferred to a microcentrifuge tube. The sample was then heat treated at 90-100 °C for 5 (±1) minutes and stored frozen at ≤ -10 °C.

SDS-PAGE Analysis

Maize-derived IPD072Aa protein samples were diluted as applicable in 1X LDS/DTT, heated for 5 minutes at 90-100 °C and then loaded into 4-12% Bis-Tris gels. Prestained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into each gel to provide a visual verification that migration was within the range of the predicted molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES running buffer and NuPAGE Antioxidant at a constant 200 volts (V) for 35 minutes. Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for N-terminal amino acid sequencing and peptide mapping.

Microbially derived lyophilized IPD072Aa protein samples were solubilized in 1X LDS sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water) and heated at 90-100 °C for 5 minutes prior to SDS-PAGE analysis. The prepared protein samples were analyzed using 4-12% Bis-Tris gels. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into the gels to provide a visual verification that migration was within the expected range of the predicted molecular weight. Electrophoresis was conducted using a Mini-Cell Electrophoresis System with 1X MES running buffer at a constant 200 volts (V) for 35 minutes. Upon completion of electrophoresis, the gels were removed from the gel cassettes and

used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for peptide mapping.

For Coomassie staining, gels were washed with water three times for a minimum of 5 minutes each, and stained with GelCode Blue Stain Reagent for approximately 60 minutes. Following staining, the gel was de-stained with water four times for a minimum of 5 minutes each or until the gel background was clear. Proteins were detected as blue-colored bands on the gels. The gel image was captured electronically using an imaging system.

Western Blot Analysis

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

Following protein transfer, the membrane was blocked in phosphate-buffered saline containing polysorbate 20 (PBST) with 5% weight/volume (w/v) non-fat dry milk for approximately 60 minutes at ambient temperature. Before and after the blocking step, the membrane was washed with PBST three times for 1-5 minutes each to reduce the background. The blocked membrane was incubated in an IPD072Aa polyclonal antibody R2409 (Pioneer Hi-Bred International, Inc.) diluted either 1:5,000 or 1:10,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient temperature. Following primary antibody incubation, the membrane was washed with PBST three or four times for 5 minutes each. The membrane was incubated in a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient temperature. The membrane was then washed with PBST three or four times for 5 minutes each. The blot remained in PBST prior to incubating with a chemiluminescent substrate for 5 minutes. The chemiluminescent signal and the pre-stained markers were detected and captured using an imaging system.

Peptide Mapping by Mass Spectrometry

Following SDS-PAGE, Coomassie staining, and imaging of the gels, the IPD072Aa protein band was excised from each sample lane and prepared for peptide mapping analysis.

Maize-derived IPD072Aa protein samples were reduced with DTT, alkylated with iodoacetamide, and then subsequently digested with trypsin or chymotrypsin. The digested samples were separated on a nanoACQUITY UPLC (Waters Corporation) fitted with a Peptide BEH C18 300 Å 1.7 µm column (75 µm x 100 mm; Waters Corporation) by gradient elution. Eluent from the column was directed into an electrospray source, operating in positive mode, on a TripleTOF 5600+ hybrid quadrupole-TOF mass spectrometer (AB Sciex; currently Sciex). The resulting MS data were processed using MS Data Converter to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software version 2.8.0) and match peptides from the expected IPD072Aa protein sequence (Perkins et al., 1999). The following search parameters were used: peptide and fragment mass tolerance, ± 0.1 Da; fixed modifications, cysteine carbamidomethyl; variable modifications, methionine oxidation; maximum missed cleavages, 1 for trypsin and 2 for chymotrypsin. The Mascot-generated peptide ion score threshold was >13 which indicates identity or extensive homology ($p<0.05$). The combined sequence coverage was calculated with GPMAW version 12.10.0.

Microbially derived IPD072Aa protein samples were sent to Alphalyse for peptide sequencing. The protein samples were reduced and alkylated with iodoacetamide (i.e., carbamidomethylated), and subsequently digested in

chymotrypsin which cleaves after leucine, phenylalanine, tryptophan, and tyrosine residues. The resulting peptides were analyzed on a Bruker Autoflex Speed MALDI TOF/TOF instrument in positive reflector mode for accurate peptide mass determination. MALDI MS/MS was performed on some peptides for peptide fragmentation analysis, i.e., partial sequencing. The MS and MS/MS spectra were combined and used for database searching using the Mascot software.

N-Terminal Amino Acid Sequencing Analysis

Maize-Derived IPD072Aa Protein: Following SDS-PAGE, the resulting gel was incubated in cathode buffer (60 mM Tris, 40 mM CAPS, 0.075% SDS, pH 9.6) for 10-20 minutes. An Immobilon-P^{SQ} PVDF membrane was wetted in 100% methanol for 30 seconds, followed by immersion in anode buffer (60 mM Tris, 40 mM CAPS, 15% methanol, pH 9.6) for 10-20 minutes. A Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system was used to transfer proteins from the gel to the membrane at 12 V for 45 minutes. Following protein transfer, the membrane was was washed with water three times for 5 minutes each wash, stained with GelCode Blue stain reagent for 5 minutes, and then destained with water to visualize the IPD072Aa protein band. A band containing the maize-derived IPD072Aa protein was excised and stored frozen (-20 °C freezer unit). The sample was analyzed using a Shimadzu PPSQ-51A sequencer. Ten cycles of Edman sequencing were performed. During each cycle, the N-terminal amino acid was sequentially derivatized with phenylisothiocyanate (PITC), cleaved with trifluoracetic acid, and converted to PTH-amino acid which was identified through chromatography. LabSolutions Software was used to automatically identify the N-terminal sequence.

Microbially Derived IPD072Aa Protein: the lyophilized IPD072Aa protein samples were solubilized in a solution of 0.5 mM ammonium acetate and 3% methanol, and sent to Alphalyse for Edman N-terminal amino acid sequencing using an ABI Procise 494 sequencer (Applied Biosystems, Inc.) equipped with an online high performance liquid chromatography system.

Glycoprotein Analysis

A Pierce Glycoprotein Staining Kit was used to determine whether the IPD072Aa protein was glycosylated. The IPD072Aa protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor) were run by SDS-PAGE as described above.

Following electrophoresis, the gel was washed with water twice for 5 minutes each wash, fixed with 50% methanol for 30-35 minutes, and washed twice with 3% acetic acid for 10-15 minutes each wash. The gel was then incubated with oxidizing solution for 15-20 minutes and washed three times with 3% acetic acid for 5-7 minutes each wash. The gel was incubated with glycoprotein staining reagent for 15-20 minutes and then incubated in a reducing reagent for 5-7 minutes. The gel was then washed one to three times with 3% acetic acid for 5-7 minutes each wash and then rinsed in water once for 5 minutes. Glycoproteins were detected as magenta-colored bands on the gel.

Following glycoprotein detection, the image of the gel was captured electronically. The same gel was then stained with GelCode Blue stain reagent for approximately 60 minutes followed by three washes with water (minimum 5 minutes each wash) to visualize all protein bands. The image of the GelCode stained gel was then captured electronically.

APPENDIX E. METHODS FOR CHARACTERISATION OF PAT PROTEIN (ET AL., 2022 (PHI-2022-055 STUDY))

Test Substance

The reference substance consisted of lyophilized PAT protein derived from a microbial expression system (lot number PCF-0038). A sample of the PAT reference substance (lot PCF-0038) was solubilized in 1X LDS/DTT (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% ultrapure [American Society for Testing and Materials (ASTM) Type 1] water [referred to as water]), then heated at 90-100 °C for 5 minutes. The sample was stored frozen (-20 °C freezer unit).

The test substance consisted of PAT protein isolated from whole plant tissue derived from DP51291 maize. The whole plant tissue was collected at the V9 growth stage (the stage when the collar of the ninth leaf becomes visible; Abendroth et al., 2011) of development from plants grown at a field location in Johnston, IA, USA. The tissue was lyophilized, homogenized and stored at ≤ -50 °C.

Protein Extraction, Purification, and Concentration

The PAT protein was extracted from lyophilized maize tissue by homogenization with a pre-chilled Waring blender vessel using phosphate-buffered saline containing polysorbate 20 (PBST) extraction buffer (approximately 25 g of plant tissue per 500 ml of extraction buffer). The sample extract was then filtered through cheesecloth and clarified by centrifugation. Ammonium sulfate (AS) precipitation was used to further purify and concentrate the sample extract. Beginning at 0% AS saturation, AS was slowly added to the sample extract while stirring until 45% AS saturation was reached. The sample was centrifuged and additional AS was added to the supernatant until 60% saturation. The sample was centrifuged again, the supernatant was discarded, and the fractionated pellets were solubilized in phosphate-buffered saline (PBS), and buffer-exchanged using Econo-Pac 10DG columns from BioRad.

The buffer-exchanged sample was further purified by immunoaffinity chromatography. The immunoaffinity column was prepared by coupling a PAT monoclonal antibody (2C10.D5.G8) to AminoLink Plus Coupling Resin. The buffer-exchanged 45-60% AS cut sample was diluted 1:4 in PBS and loaded to the column. The sample passed through the column was collected and loaded back to the column once. The column was then washed with 20 column volume (CV) PBST. The PAT protein was eluted off the column using IgG elution buffer. Fractions (1 CV each) were collected separately and immediately neutralized with 0.1 CV of 1M Tris buffer, pH 8.

The PAT protein was further purified by ion exchange purification using a Q Sepharose column. Eluted fractions 2-4 from each immunoaffinity purification column were pooled, diluted in 50 mM Tris buffer, pH 8, and then added to the column containing the Q Sepharose resin. The PAT protein was eluted off the Q Sepharose column using 50 mM Tris buffer, pH 8, with 500 mM sodium chloride. Collected fractions from elutions 2-5 (1 CV each) were pooled and concentrated using a centrifugal concentrator (10K Vivaspin; Sartorius) and buffer-exchanged to a volume of approximately 200 µl. NuPAGE LDS Sample Buffer and 10X NuPAGE Sample Reducing Agent were added to the sample in the concentrator to final concentrations of 25% and 10%, respectively. The sample was heated at 70 100 °C for 2 5 minutes, transferred to a snap-top tube, heated at 90 100 °C for 5 (±1) minutes, and stored frozen (20 °C freezer unit).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The partially purified maize-derived PAT protein sample was diluted as applicable in 1X LDS/DTT, heated at 90-100 °C for 5 minutes, and then loaded into 4-12% Bis-Tris gels. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into each gel to provide a visual verification that migration was within the expected range of the predicted molecular weight. In addition, the diluted PAT protein reference substance was diluted in 1X LDS/DTT as appropriate, heated at 90-100 °C for 5 minutes, and loaded into each gel. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES running buffer and NuPAGE Antioxidant at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, protein glycosylation analysis, sample preparation for peptide mapping, or protein transfer to a membrane for western blot analysis and N-terminal amino acid sequencing.

Gels for Coomassie staining were washed with water three times for 5 minutes each wash and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, gels were de stained with water four times for at least 5 minutes each until the gel background was clear. Proteins were stained as blue colored bands on the gel. The gel image was captured electronically using a ChemiDoc MP imaging system (Bio Rad).

Western Blot Analysis

Following SDS-PAGE, a gel was assembled into a nitrocellulose (NC) iBlot Gel Transfer Stack. An iBlot Gel Transfer Device was used to transfer proteins from the gel to the NC membrane for 7 minutes with a pre-set program (P3). Following protein transfer, the membrane was blocked in PBST containing 5% weight/volume (w/v) non-fat dry milk for 60 minutes at ambient laboratory temperature. Before and after the blocking step, the membrane was washed with PBST three times for 1 minute each wash to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with a PAT monoclonal antibody 22H2.G4 (Pioneer) diluted 1:5000 in PBST containing 1% w/v non-fat dry milk. Following primary antibody incubation, the membrane was washed in PBST three times for 5 minutes each wash. The membrane was incubated with a secondary antibody (anti-mouse IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient laboratory temperature. The membrane was washed with PBST three times for 5 minutes each wash. The membrane 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 a ChemiDoc MP imaging system

Peptide Mapping and Sequencing Analysis by LC-MS Analysis

For peptide mapping, SDS-PAGE, Coomassie staining, and gel imaging were performed using the methods as described above. PAT protein bands were excised from the gel and stored frozen (-20 °C freezer unit). The protein in two of the gel slices was reduced with DTT, alkylated with iodoacetamide, and then subsequently digested with trypsin or chymotrypsin. The digested samples were separated on a nanoACQUITY UPLC (Waters Corporation) fitted with a Peptide BEH C18 300 Å 1.7 µm column (75 µm x 100 mm; Waters Corporation) by gradient elution. Eluent from the column was directed into an electrospray source, operating in positive mode, on a TripleTOF 5600+ hybrid quadrupole-TOF mass spectrometer (AB Sciex; currently Sciex). The resulting mass spectrometry (MS) data were processed using MSConverter to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software version 2.8.0) and match peptides from the expected PAT protein sequence (Perkins *et al.*, 1999). The following search parameters were used: peptide and fragment mass tolerance, ± 0.1 Da; fixed modifications, cysteine carbamidomethyl; variable modifications, methionine oxidation; and maximum missed cleavages, 1 for

trypsin and 2 for chymotrypsin. The Mascot-generated peptide ion score threshold was >13, which indicates identity or extensive homology ($p < 0.05$). The combined sequence coverage was calculated with GPMAW version 12.10.0.

N-Terminal Amino Acid Sequence Analysis

Following SDS-PAGE, as described above, the resulting gel was incubated in cathode buffer (60 mM Tris, 40 mM CAPS, 0.075% SDS, pH 9.6) for 10-20 minutes. An Immobilon-PSQ PVDF membrane was wetted in 100% methanol for 1 minute, followed by immersion in anode buffer (60 mM Tris, 40 mM CAPS, 15% methanol, pH 9.6) for 10-20 minutes. A Trans-Blot SD Semi Dry Electrophoretic Transfer Cell system was used to transfer proteins from the gel to the membrane at 10 V for 60 minutes. Following protein transfer, the membrane was washed with water three times for 5 minutes each wash, stained with GelCode Blue stain reagent for 5 minutes, and then destained with water or a mixture of water and 50% methanol to visualize the PAT protein. Duplicate bands containing the maize-derived PAT protein were excised and stored frozen (-20 °C freezer unit). The gel slices were analyzed as a single sample using a Shimadzu PPSQ-51A sequencer. Ten cycles of Edman sequencing were performed. During each cycle, the N-terminal amino acid was sequentially derivatized with phenylisothiocyanate (PITC), cleaved with trifluoracetic acid, and converted to PTH amino acid which was identified through chromatography. LabSolutions Software was used to automatically identify the N-terminal sequence.

Protein Glycosylation Analysis

The Pierce Glycoprotein Staining Kit was used to determine whether the PAT protein was glycosylated. The purified maize-derived PAT protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor) were run by SDS PAGE as described above.

Following electrophoresis, the gel was washed with water twice for 5 minutes each wash, fixed with 50% methanol for 30-35 minutes, and washed twice with 3% acetic acid for 10-15 minutes each wash. The gel was then incubated with oxidizing solution for 15-20 minutes and washed three times with 3% acetic acid for 5-7 minutes each wash. The gel was incubated with glycoprotein staining reagent for 15-20 minutes and then incubated in a reducing reagent for 5-7 minutes. The gel was then washed with 3% acetic acid three times for 5 minutes each wash and then rinsed in water for 5 minutes. Glycoproteins were detected as bands stained a magenta color on the gel.

Following glycoprotein detection, the image of the gel was captured electronically using a ChemiDoc MP imaging system. The same gel was then stained with GelCode Blue Stain Reagent for 60 minutes and destained with water three times for at least 5 minutes each to visualize all protein bands. The image of the GelCode stained gel was then captured electronically.

APPENDIX F. METHODS FOR CHARACTERISATION OF PMI PROTEIN (ET AL., 2022 (PHI-2022-123 STUDY))

Test Materials

The reference substance consisted of lyophilized PMI protein derived from a microbial expression system (lot number PCF-0055). A 5.0-mg sample of the lyophilized protein was solubilized in 3.4 ml of 1X LDS/DTT sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% water) to a target concentration of 1 mg PMI protein/ml and heated at 90-100 °C for 5 minutes.

The test substance consisted of PMI protein isolated from whole plant tissue derived from DP51291 maize. The whole plant tissue was collected at the V9 growth stage (the stage when the collar of the ninth leaf becomes visible; Abendroth *et al.*, 2011) of development from plants grown at a Pioneer owned field location (Johnston, IA, USA). The tissue was lyophilized, homogenized and stored at ≤ -50 ° C.

Protein Extraction, Purification, and Concentration

The PMI protein was extracted from lyophilized maize tissue by homogenization in a pre-chilled Waring blender vessel using phosphate-buffered saline containing polysorbate 20 (PBST) extraction buffer with protease inhibitor. The sample extract was then filtered through cheesecloth, clarified by centrifugation, and fractionated using ammonium sulfate (AS) precipitation. Beginning at 0% AS saturation, AS was slowly added to the sample extract while stirring until 45% AS saturation was reached. The sample was centrifuged and the AS process was repeated with the supernatant, using the 45% AS saturation and progressing to 60%. The sample was centrifuged again, the supernatant was discarded, and the fractionated pellets were solubilized and buffer-exchanged in phosphate-buffered saline using Econo-Pac 10DG desalting columns (BioRad).

Following buffer exchange, the sample was further purified by immunoaffinity chromatography. The immunoaffinity columns were prepared by coupling a PMI polyclonal antibody (R164; Pioneer) to AminoLink Plus Coupling Resin. The PMI protein sample was eluted off the column using IgG Elution buffer. Elutions 2-5 from the immunoaffinity purification column were collected separately. The elutions were then concentrated using a centrifugal concentrator (30K Vivaspin Turbo 4; Sartorius) to a volume of 2 ml. The PMI protein was further purified by ion exchange purification using a HiTrap Q HP ion exchange resin column (Cytiva). The PMI sample eluted from HiTrap Q HP column was concentrated and buffer exchanged to 50 mM Tris buffer, pH 8.

Following extraction, purification, and concentration, the final volume of the PMI sample was estimated to be approximately 100 µl and an equal volume of 2X LDS/DTT sample buffer (50% 4X NuPAGE LDS Sample Buffer, 20% 10X NuPAGE Sample Reducing Agent containing DTT, and 30% ASTM [American Society for Testing and Materials] Type I water [referred to as water]) was added to the concentrated sample. The sample was transferred to a microcentrifuge tube, heat treated at 90-100 °C for 5 (±1) minutes, and stored frozen (-20 °C freezer unit).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The partially purified maize-derived PMI protein sample was re-heated for 5 minutes at 90-100 °C, diluted as applicable, and then loaded into 4-12% Bis-Tris gels. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were loaded into each gel to provide a visual verification that migration was within the

expected range of the predicted molecular weight. For SDS PAGE and western blot analysis, the PMI protein reference substance was diluted in 1X LDS/DTT to approximately the same concentration as the maize-derived protein, re heated for 5 minutes at 90-100 °C, and loaded into the gel. Electrophoresis was conducted using a pre-cast gel electrophoresis system with MES running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for peptide mapping.

For Coomassie staining, the gel was washed with water three times for 5 minutes each wash and stained with GelCode Blue Stain Reagent for 60-90 minutes. Following staining, the gel was de stained with water four times for at least 5 minutes each until the gel background was clear. Proteins were stained as blue colored bands on the gel. The gel image was captured electronically using a ChemiDoc MP (Bio Rad) imaging system.

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 one-three times for at least 1 minute each wash to reduce the background. The blocked membrane was incubated with a PMI monoclonal antibody (13D11.F11.C12; Pioneer) conjugated to horseradish peroxidase diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk for 60 minutes at ambient laboratory temperature. The membrane was washed with PBST three times for 5 minutes each wash. The membrane 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 a ChemiDoc MP imaging system.

Peptide Mapping and N-Terminal Amino Acid Sequence Analysis by LC/MS

Following SDS-PAGE, Coomassie staining, and gel imaging using the methods as described above, bands containing the PMI protein were excised from a gel and stored frozen at ≤ 5 °C. The protein in each gel slice was reduced with DTT, alkylated with iodoacetamide, and then subsequently digested with trypsin or chymotrypsin. The digested samples were separated on a nanoACQUITY UPLC (Waters Corporation) fitted with a Peptide BEH C18 300 Å 1.7 µm column (75 µm x 100 mm; Waters Corporation) by gradient elution. Eluent from the column was directed into an electrospray source, operating in positive ion mode, on a TripleTOF 5600+ hybrid quadrupole-TOF mass spectrometer (AB Sciex; currently Sciex). The resulting mass spectrometry (MS) data were processed using MSConverter to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software version 2.8.0) and match peptides from the expected PMI protein sequence (Perkins *et al.*, 1999). The following search parameters were used: peptide and fragment mass tolerance, ± 0.1 Da; fixed modifications, cysteine carbamidomethyl; variable modifications, acetyl (protein N-terminal), methionine oxidation; and maximum missed cleavages, 1 for trypsin and 2 for chymotrypsin. The Mascot-generated peptide ion score threshold was > 13 which indicates identity or extensive homology ($P < 0.05$). The combined sequence coverage was calculated with GPMAW version 12.10.0.

Protein Glycosylation Analysis

The Pierce Glycoprotein Staining Kit was used to determine whether the PMI protein was glycosylated. The partially purified maize-derived PMI protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor) were run by SDS PAGE as described above.

Following electrophoresis, the gel was washed with water twice for at least 5 minutes each wash, fixed with 50% methanol for 30-35 minutes, and washed twice with 3% acetic acid for 10-15 minutes each wash. The gel was then incubated with oxidizing solution for 15-20 minutes and washed with 3% acetic acid three times for 5-7 minutes each wash. The gel was incubated with glycoprotein staining reagent for 15-20 minutes and then incubated in a reducing reagent for 5-7 minutes. The gel was then washed with 3% acetic acid for 5 minutes and then washed in water for 5 minutes. Glycoproteins were detected as bands stained a magenta color on the gel.

Following glycoprotein detection, the image of the gel was captured electronically using a ChemiDoc MP imaging system. The same gel was then stained with GelCode Blue stain reagent for 60 minutes followed by three washes with water for 5 minutes each wash to visualize all protein bands. The image of the GelCode stained gel was then captured electronically.

APPENDIX G. METHODS FOR EXPRESSED TRAIT ANALYSES ([redacted] ET AL., 2022 (PHI-2021-034 STUDY))

Field Trial Experimental Design

A multi-site field trial was conducted during the 2021 growing season at six sites in commercial maize-growing regions of the United States (one site in Iowa, Illinois, Indiana, Pennsylvania, and Texas) and Canada (one site in Ontario). A randomized complete block design with four blocks was utilized at each site. Procedures employed during the field trial to control the introduction of experimental bias included randomization of maize entries within each block and uniform maintenance treatments across each plot area. An herbicide treatment of glufosinate-ammonium was applied to DP51291.

Sample Collection

The following tissue samples were collected: root (V6, V9, R1, and R4 growth stage), leaf (V9, R1, and R4 growth stages), pollen (R1 growth stage), forage (R4 growth stage), and grain (R6 growth stage). Growth stages are described in Table G1. One sample per plot was collected for each tissue set at the applicable growth stages. All samples from a given growth stage were collected from the same plants. All samples from R4 and R6 growth stages were collected from self-pollinated plants. All samples were collected from impartially selected, healthy, representative plants to minimize potential bias.

Table G1. 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).

Samples were collected as follows:

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

- Each leaf sample was obtained by pruning the youngest leaf that emerged at least 8 in. (20 cm) in length. The tissue was cut into sections of 1 in. (2.5 cm) or less in length and collected into a pre labeled vial.
- Each pollen sample was obtained by bagging and shaking a selected tassel to dislodge the pollen. The tassel selected for sampling had one-half to three-quarters of the tassel's main spike shedding pollen. For some plots, pollen may have been pooled from multiple plants within the same plot in order to collect the appropriate amount. The pollen was screened for anthers and foreign material, and then collected to fill approximately 25-50% of the conical area of a pre-labeled vial.
- Each forage sample was obtained by cutting the plants approximately 4-6 in. (10-15 cm) above the soil surface. Samples were composed of all above-ground plant parts, including the stalk, leaves, self pollinated ear (husks, grain, and cob), and tassel. Leaves were cut into sections that were 12 in. (30 cm) or less in length and all other plant parts were cut into sections that were 3 in. (7.6 cm) or less in length. After tissues were cut to the appropriate lengths, samples were placed in pre-labeled, plastic-lined cloth bags. Samples were collected at the R4 growth stage and included all above ground plant parts in order to produce samples that are representative of maize forage (whole aerial plant including grain harvested at high moisture content) that is fed to animals as silage.
- Each grain sample was obtained by husking and shelling the grain from one primary ear that had previously been self-pollinated. For each sample, a representative sub sample of 10 kernels was collected into an individual pre-labeled vial.

Sample Processing, Shipping, and Storage

Each sample was uniquely labeled with a sample identification number and barcode for sample tracking by site, entry, block, tissue, and growth stage. Each sample was placed on dry ice within 10 minutes of collection in the field and transferred to frozen storage (-20 °C freezer unit) until shipment. Expressed trait protein samples were then shipped frozen to Pioneer Hi Bred International, Inc. for processing and analysis. Upon arrival, samples were stored frozen (-20 °C freezer unit). Forage samples were coarsely homogenized prior to lyophilization. All samples were lyophilized under vacuum until dry. Following lyophilization, pollen samples were stored frozen until analysis and leaf, root, forage, and grain samples were finely homogenized and stored frozen until analysis.

Protein Concentration Determination

The concentrations of IPD072Aa, PAT, and PMI proteins were determined using quantitative enzyme linked immunosorbent assay (ELISA) methods that have been internally validated to demonstrate method suitability.

Processed tissue sub samples were weighed at the following target weights: 5 mg for pollen; 10 mg for leaf; 20 mg for grain and root; and 30 mg for forage. Pollen, leaf, grain, and forage samples analyzed for IPD072Aa protein were extracted with 0.60 ml of chilled 25% StabilZyme Select in phosphate buffered saline containing polysorbate 20 (PBST). Root samples analyzed for IPD072Aa protein were extracted in chilled H5 buffer, which was comprised of 90 mM HEPES, 140 mM sodium chloride, 1.0% polyethylene glycol, 1.0% PVP 40, 1.0% bovine serum albumin, 0.007% thimerosal, and 0.3% polysorbate 20. Samples analyzed for PAT and PMI proteins were extracted with 0.60 ml of chilled PBST. All extracted samples were centrifuged, and then supernatants were removed and prepared for analysis.

ELISA Methods

ELISA methods were performed as follows:

- IPD072Aa Protein ELISA Method: Prior to analysis, samples were diluted as applicable with 25% StabilZyme Select in PBST. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre coated with an IPD072Aa specific antibody. Following incubation, unbound substances were washed from the plate and the bound IPD072Aa protein was incubated with a different IPD072Aa specific antibody conjugated to the enzyme horseradish peroxidase (HRP). Unbound substances were washed from the plate. Detection of the bound IPD072Aa antibody complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader.

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

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

Calculations for Determining IPD072Aa, PAT, and PMI Protein Concentrations

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

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

The 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

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

Sample Concentration = Sample x Extraction Buffer Volume (ml)

(ng protein/mg sample weight) Concentration (ng/ml) ——— Sample Target Weight (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

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

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

Statistical Analysis

Statistical analysis of the IPD072Aa, PAT, and PMI protein concentration results consisted of the calculations of means, ranges, and standard deviations. Individual sample results below the LLOQ were assigned a value equal to half of the LLOQ for calculation purposes.

APPENDIX H. METHODS FOR NUTRIENT COMPOSITION ANALYSIS (ET AL., 2019B (PHI-2021-035/021 STUDY))

Field Trial Experimental Design

A multi-site field trial was conducted during the 2021 growing season at eight 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). Each site included DP51291 maize, control maize, and four of the following non-GM commercial maize lines: 5433, H3257, K-0204, P0574, 205-17, 207-27, G07F23, P0843, P1093, 5858, H3394, K-0608, 209-50, 6076, 6046, G10T63, G11A33, 6282, G12W66, and 6269 maize (collectively referred to as reference maize). A randomized complete block design with four blocks was utilized at each site. A herbicide treatment of glufosinate-ammonium was applied to DP51291 maize. Procedures employed during the field trial to control the introduction of experimental bias included randomization of maize entries within each block and uniform maintenance treatments across each plot area.

Sample Collection

One forage sample (R4 growth stage) and one grain sample (R6 growth stage) were collected from each plot. Each forage sample (combination of three plants) was obtained by cutting the aerial portion of the plants from the root system approximately 4-6 in. (10-15 cm) above the soil surface line; the plants were chopped into sections of 3 in. (7.6 cm) or less in length, pooled, and approximately one-third of the chopped material was collected in a pre-labeled, plastic-lined, cloth bag. Each grain sample was obtained from five ears at typical harvest maturity from self-pollinated plants; the ears were husked and shelled, and the pooled grain was collected into a large, plastic, resealable bag and then placed into a pre-labeled, plastic-lined, cloth bag.

All samples were collected from impartially selected, healthy, representative plants to minimize potential bias. Reference maize and control maize samples were collected prior to the collection of DP51291 maize samples to minimize the potential for contamination. Each sample was uniquely labeled with a sample identification number and barcode for sample tracking, and is traceable by site, entry, block, tissue, and growth stage. Samples were placed into chilled storage (e.g., coolers with wet ice, artificial ice, or dry ice) after collection and, within three hours of collection, transferred to a freezer (≤ -10 °C) or placed on dry ice. Samples were shipped frozen from each site to) for nutrient composition analyses.

Nutrient Composition Analyses

The forage and grain samples were analyzed at . Experimental bias was controlled through the use of the same sample identification numbers assigned to the originally collected samples, the use of pre-set data acceptability criteria, sample randomization prior to homogenization, and through the arrangement of samples for analyses without consideration of sample identity. The following nutrient composition analytes were determined:

- *Forage proximate, fiber, and mineral composition*: moisture*, crude protein, crude fat, crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, calcium, and phosphorus
    - *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.
- *Grain proximate and fiber composition*: moisture, crude protein, crude fat, crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), ash, and carbohydrates

- *Grain fatty acid composition*: 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), and lignoceric acid (C24:0)
- *Grain amino acid composition*: alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine
- *Grain mineral composition*: calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc
- *Grain vitamin composition*: β-carotene, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B9 (folic acid), α-tocopherol, β-tocopherol, γ-tocopherol, and 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
- *Grain secondary metabolite and anti-nutrient composition*: *p*-coumaric acid, ferulic acid, furfural, inositol, phytic acid, raffinose, and trypsin inhibitor

Nutrient composition analytical methods and procedures are summarized in Table I.1.

Table I1. Methods for Compositional Analysis

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

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

Nutritional Analyte	Method
Pantothenic Acid (Vitamin B5)	The analytical procedure for the determination of pantothenic acid (vitamin B5) was based on a method from AOAC International. Pantothenic acid (vitamin B5) was determined using a microbiological assay. Pantothenic acid (vitamin B5) was extracted from the sample by adding an acetic acid buffer solution and autoclaving. The pH was adjusted and a tube array was prepared using three different dilutions of the samples. This tube array was inoculated with *Lactobacillus plantarum* and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of D-pantothenic acid hemicalcium salt.
Pyridoxine (Vitamin B6)	The analytical procedure for the determination of pyridoxine (vitamin B6) was based on a method from the AACC. Pyridoxine (vitamin B6) was determined using a microbiological assay. Pyridoxine (vitamin B6) was extracted from the sample by adding sulfuric acid and autoclaving. The pH was adjusted and a tube array was prepared using four different dilutions of the samples. This tube array was inoculated with *Saccharomyces cerevisiae* and allowed to incubate for approximately 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 600 nm. The absorbance readings were compared to a standard curve generated using known concentrations of pyridoxine hydrochloride.
Total Folate as Folic Acid (Vitamin B9)	The analytical procedure for determination of total folate as folic acid was based on a microbiological assay published by the AACC. Samples were hydrolyzed and digested by protease and amylase enzymes to release the folate from the grain. A conjugase enzyme was used to convert the naturally occurring folypolyglutamates. 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.
Total Tocopherols	The analytical procedure for determination of tocopherols was based on methods from the *Journal of the American Oil Chemists' Society* and *Analytical Sciences*. Alpha, beta, gamma, and delta tocopherols were extracted with hot hexane and the extracts were analyzed by normal phase UPLC with fluorescence detection.
β-Carotene	The analytical procedure for determination of beta-carotene was based on a method published by AOAC International. Samples were extracted using a 40:60 acetone:hexane with tert-butylhydroquinone (TBHQ) solution then analyzed by HPLC-UV.
Trypsin Inhibitor	The analytical procedure for the determination of trypsin inhibitor was based on a method published by the AOCS. Trypsin inhibitor was extracted with sodium hydroxide. Trypsin was added to the extracts to react with trypsin inhibitor. The residual trypsin activity was measured with a spectrophotometer using the chromogenic trypsin substrate Benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA). The amount of trypsin 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 analyzed for furfural content by reverse phase UPLC with UV detection.
p-Coumaric and Ferulic Acid	The analytical procedure for the determination of *p*-coumaric and ferulic acids was developed based on methods published in *Journal of Agricultural and Food Chemistry* and *The Journal of Chemical Ecology*. Ground maize grain was analyzed to determine the amounts of *p*-coumaric

Nutritional Analyte	Method
	acid and ferulic acid by separating the total content of phenolic acids using reverse phase UPLC and UV detection.
Phytic Acid	The analytical procedure for the determination of phytic acid was based on a method published by AOAC International. The samples were analyzed to determine the amount of phytic acid by extracting the phytic acid with dilute hydrochloric acid (HCl) and isolating it using an aminopropyl silica solid phase extraction column. Once isolated and eluted, the phytic acid was analyzed for elemental phosphorus by ICP-OES.

Statistical Analysis of Nutrient Composition Data

Prior to statistical analysis, the data were processed as follows:

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

- *Conversion of fatty acid assay values*: The raw data for all fatty acid analytes were provided by [redacted] 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.

- *Calculation of additional analytes*: One additional analyte (total tocopherol) was calculated for statistical analyses. The total amount of tocopherol for each sample was obtained by summing the assay values of α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol in the sample. If the assay value of an individual analyte was below the LLOQ for a given sample, half of the LLOQ value was used in computing the total. The total was considered below the LLOQ only when all the individual analytes contributing to its calculation were below the LLOQ.

Statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute, Inc.). The following rules were implemented for each analyte:

- If both DP51291 maize and the control maize had < 50% of samples below the LLOQ, then an across-site mixed model analysis would be conducted.

- If, either DP51291 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 DP51291 maize and the control maize had 100% of samples below the LLOQ, then statistical analyses would not be performed.

Statistical Model for Across-Site Analysis

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

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

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

where μ_i denotes the mean of the ith entry (fixed effect), ℓ_j denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth 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 ith entry in the kth block of the jth site (random effect or residual). Notation ~ $iid\ N(0, \sigma^2_a)$ 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 DP51291 maize and the control maize. The Kenward-Roger method (Kenward and Roger, 2009) was used to estimate the variance of LS-Means and approximate degrees of freedom for statistical tests. 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 among sites.

False Discovery Rate Adjustment

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

Interpretation of Statistical Results

For a given analyte, when a statistically significant difference (P-value from mixed model analysis < 0.05, or Fisher's exact test P-value < 0.05) was identified in the across-site analysis, the respective range of individual values from DP51291 maize was compared to a tolerance interval. Tolerance intervals are expected to contain at least 99% of the values for corresponding analytes of the conventional maize population with a 95% confidence level (Hong *et al.*, 2014). The tolerance intervals were derived from Pioneer and Dow AgroSciences proprietary accumulated data from non-GM maize lines, which were grown in commercial maize-growing regions between 2003 and 2020 in the United States, Canada, Chile, Brazil, and Argentina. The combined data represent 196 commercial maize lines and 202 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 DP51291 maize contained individual values outside the tolerance interval, it was then compared to the respective literature range obtained from published literature (AFSI, 2022; Codex Alimentarius Commission, 2019; Cong *et al.*, 2015; Lundry *et al.*, 2013; OECD, 2002; Watson, 1982). Literature ranges complement 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 Agriscience's proprietary database.

If the range of DP51291 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 complement 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.

Table I2. Number of Sample Values Below the Lower Limit of Quantification (sprayed)

Analyte	Number of Samples Below the LLOQ		Fisher's Exact Test P-Value
	Control Maize (n=32)	Herbicide-Treated DP51291 Maize (n=32)	
Fatty Acid Composition (% Total Fatty Acids)			
Lauric Acid (C12:0)	32	32	--
Myristic Acid (C14:0)	32	32	--
Palmitoleic Acid (C16:1)[a]	7	2	--
Heptadecanoic Acid (C17:0)	31	28	0.355
Heptadecenoic Acid (C17:1)	32	32	--
Eicosadienoic Acid (C20:2)	32	32	--
Behenic Acid (C22:0)	21	17	0.446
Mineral Composition (% Dry Weight)			
Copper[a]	9	6	--
Sodium[a]	2	0	--
Vitamin Composition (mg/kg Dry Weight)			
Vitamin B2 (Riboflavin)	32	32	--
α-Tocopherol[a]	3	2	--
β-Tocopherol	31	31	1.00
δ-Tocopherol	26	26	1.00
Secondary Metabolite and Anti-Nutrient Composition (% Dry Weight)			
Furfural	32	32	--
Raffinose	16	12	0.450

[a] This analyte had < 50% of sample values below the lower limit of quantification (LLOQ) in each maize line and was subjected to the mixed model analysis.