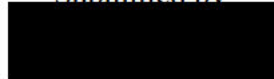
Application to Amend the Australia New Zealand Food Standards Code
Schedule 26 - *Food Produced Using Gene Technology*

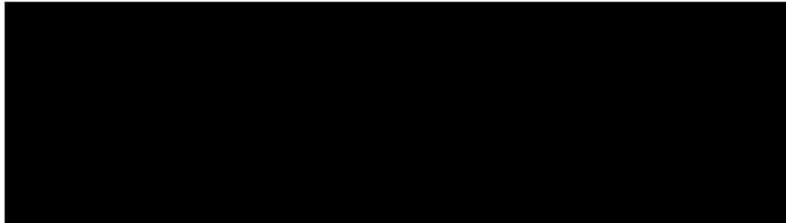
OECD Unique Identifier: DAS-Ø1131-3

DAS1131 Maize

Submitting company:
Corteva Agriscience Australia Pty Ltd

Submitted by:





June 2023

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SUMMARY

Corteva, Inc. is a publicly traded, global pure-play agriculture company that combines industry-leading innovation, high-touch customer engagement and operational execution to profitably deliver solutions for the world's most pressing agriculture challenges. Corteva generates advantaged market preference through its unique distribution strategy, together with its balanced and globally diverse mix of seed, crop protection, and digital products and services. With some of the most recognized brands in agriculture and a technology pipeline well positioned to drive growth, the company is committed to maximizing productivity for farmers, while working with stakeholders throughout the food system as it fulfills its promise to enrich the lives of those who produce and those who consume, ensuring progress for generations to come. 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 food uses of insect-resistant and herbicide-tolerant maize (*Zea mays* L.) event DAS-Ø1131-3 (referred to as DAS1131 maize), a new food produced using gene technology.

DAS1131 maize was genetically modified to express the Cry1Da2 protein for protection against certain susceptible lepidopteran pests and the DGT-28 EPSPS protein for tolerance to glyphosate herbicide. Both proteins are presented to FSANZ for review for the first time.

This application presents information supporting the safety and nutritional comparability of DAS1131 maize. The molecular characterization analyses conducted on DAS1131 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 Cry1Da2 and DGT-28 EPSPS proteins were evaluated and were found unlikely to be allergenic or toxic to humans. A compositional equivalence assessment demonstrated that the nutrient composition of DAS1131 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 DAS1131 maize containing the Cry1Da2 and DGT-28 EPSPS proteins is as safe and nutritious as non-GM maize for food uses.

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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*
☑	12	B Applicant details
☑	12	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
☑	11	K Statutory Declaration
		L Checklist/s provided with application
		☑ *3.1.1 Checklist*
☑	9	☑ *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**
☑	16	A.1 Nature and identity
☑	17	A.2 History of use of host and donor organisms
☑	19	A.3 Nature of genetic modification
☑	50	B.1 Characterisation and safety assessment
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☑	111	B.5 Compositional analyses
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STATUTORY DECLARATION

Statutory Declarations Act 1959 [1]

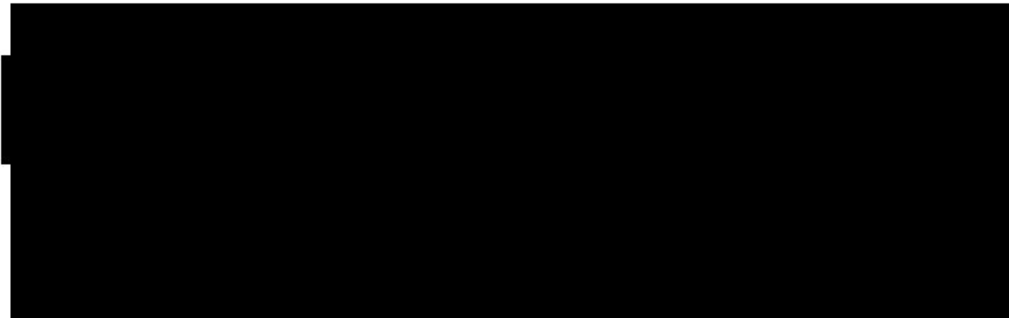
make the following declaration under the *Statutory Declarations Act 1959*:

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 on 9th of February 2022



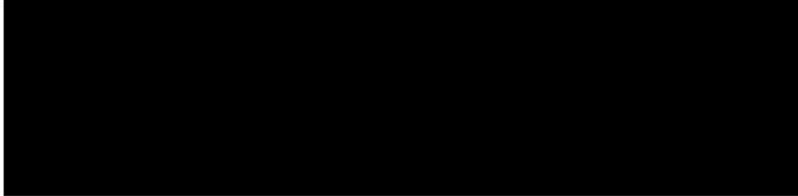
[1] http://www.comlaw.gov.au/Series/C1959A00052.

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 application is submitted by:



The primary contact is:

The Managing Director of Corteva Agriscience Australia Pty Ltd is:

Ph:
Email:

C. Purpose of the application

Corteva Agriscience Australia Pty Ltd, member of Corteva Agriscience group of companies, and its affilates entites (herein referred to collectively as Corteva), has developed DAS1131 maize (OECD Unique Identifier DAS-Ø1131-3), a new event that has been transformed to express the Cry1Da2 protein for protection against certain susceptible lepidopteran pests and the DGT-28 EPSPS protein for tolerance to glyphosate herbicide.

As a result of this application, Corteva 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 DAS1131*.

D. Justification for the application

D(a) Need for the proposed change

Corteva is a member of Excellence Through Stewardship™ (ETS). Corteva has developed the new maize event DAS1131, which is being 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. Corteva's application to amend Standard 1.5.2 with respect to DAS1131 maize is in support of these policies.

D(b) Advantage of the genetically modified food

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 DAS1131 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).

Insect Resistance

Certain lepidopteran insects are serious pests of maize in the United States. Control of lepidopteran maize pests has historically been managed with crop rotation, broad-spectrum insecticides, and transgenic crops expressing crystalline (Cry) proteins. As adoption of Bt maize has increased, the selection pressure on target insects to develop resistance has become greater. Insect resistance to transgenic traits can reduce the efficacy of the traits over time, increasing costs of maize production, and/or reducing yield.

Herbicide Tolerance

Genetically modified (GM) herbicide-tolerant maize lines are widely cultivated because they provide additional weed management options for growers. Herbicide-tolerant maize has a significant impact on growers' earnings as they can be used on an as-needed basis and can help growers to adopt reduced or no tillage practices (Fawcett and Towery, 2003; Fernandez-Cornejo et al., 2012).

The DGT-28 EPSPS protein is tolerant to glyphosate herbicide. DAS1131 maize provides farmers with an additional control option for herbicide management practices.

D.1 Regulatory impact

Corteva have developed the new maize line DAS1131, 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 approach to responsible 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 launches new products in accordance with the Corteva Product Launch Policy and Excellence Through Stewardship Product Launch Guidance. Our long-standing, multi-faceted approach includes evaluating export market information, performing value-chain consultations and consideration of regulatory functionality. Innovative technologies like DAS1131 maize are designed to deliver exceptional value and needed performance to the farmers that produce grain from these products, along with helping farmers provide enough safe, nutritious food to meet global demand. In line with these guidelines, Corteva's approach to responsible launches of new products includes a long-standing process to evaluate export market information, value chain consultations, regulatory functionality, preparedness to meet product ramp up and demand plans, and other factors. Corteva continues to advocate for a global synchronous, science-based and predictable regulatory system. We also encourage farmers, industry, and consumer groups to continue to advocate for the acceptance of new, innovative technologies that help to improve farm productivity and profitability and contribute to the global economy and environmental sustainability.

Corteva does not develop nor import food or feed products into the Australian or New Zealand markets. The proposed amendment to the Standard, however, may result in increasing Australia and New Zealand's access to international grain food markets while supporting Corteva's sale of seed in markets where DAS1131 maize is to be cultivated. In this sense, and in an effort to maintain transparency with FSANZ, Corteva acknowledges that there may be a capturable commercial benefit to Corteva as defined in Section 8 of the FSANZ Act. Any relevant local costs are made up of Corteva personnel time both locally and globally as well as of the direct fees associated with the submission.

Most of the sweet corn consumed in Australia is grown domestically. Domestic production of corn in Australia and New Zealand is supplemented by importation 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 DAS1131 maize. This offers benefits to the industry and consumers in Australia and New Zealand, which result from the advantages of DAS1131 maize available to growers in cultivation countries (see Section D(b) *Advantage of the genetically modified food of the dossier* above).

While Corteva does not possess quantitative data, which would allow it to estimate the benefits in monetary terms, DAS1131 maize is anticipated to contribute to the maintenance of stable global maize supply, choice and affordability for consumers. No specific costs associated with the approval of DAS1131 maize for Australian and New Zealand consumers have been identified.

Similarly, an analysis in monetary terms for the grain and food industry is hard to determine, however, Australian and New Zealand importers are expected to benefit from trade access, which the approval of DAS1131 maize will support (see Section D.1.2 *Impact on international trade* below). Compliance with import requirements is also anticipated to be simplified when sourcing from markets in which DAS1131 maize is commercialized as a seed product. The only identified costs associated with the approval of DAS1131 maize for Australian and New Zealand industry is meeting their GM labelling requirements for those foods derived from DAS1131 maize which trigger them, similarly to other existing GM maize varieties.

No dollar value of the costs and benefits for the governments can be assigned with the available information. However, from the government perspective, approval of DAS1131 maize will support global regulatory harmonization and limit potential instances of non-compliance related to the regulation of GM foods. No costs associated with the approval of DAS1131 maize for the Australian and New Zealand governments have been identified.

D.1.2 Impact on international trade

The addition of DAS1131 maize to Schedule 26 is anticipated to facilitate import access to maize from the applicable cultivation countries. Without such an approval, grain handlers may undertake a scientifically unnecessary and costly activities to segregate DAS1131 maize and food products derived from it for Australian and New Zealand markets. Therefore, amending the Food Code to include DAS1131 maize is anticipated to have a positive impact on Australian and New Zealand access to international commodity trade markets.

A. TECHNICAL INFORMATION ON THE FOOD PRODUCED USING GENE TECHNOLOGY

A.1 Nature and identity of the genetically of the genetically modified food

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

DAS1131 maize was genetically modified to produce the Cry1Da2 protein for protection against certain susceptible lepidopteran pests and the DGT-28 EPSPS protein for tolerance to glyphosate herbicide.

The Cry1Da2 protein is encoded by the *cry*1Da2 gene, a chimeric gene comprised of sequences from the *cry*1Da2 gene encoding an insecticidal core toxin and a derivative of the *cry*1Ab gene, both derived from *Bacillus thuringiensis* (*Bt*). The expressed Cry1Da2 protein binds to receptors in the brush border membrane of certain susceptible lepidopteran pests and causes cell death through the formation of non-specific, ion conducting pores in the apical membrane of the midgut epithelial cells.

The DGT-28 EPSPS protein is encoded by the *dgt-28 epsps* (5-enolpyruvylshikimate-3-phosphate synthase) gene derived from *Streptomyces sviceus,* fused to a chimeric chloroplast transit peptide, TraP8, from *Brassica napus* and *Brassica rapa.* The expressed DGT-28 EPSPS protein is targeted to the maize chloroplasts through the TraP8 peptide to provide tolerance to glyphosate herbicide. Other natural and modified EPSPS proteins have been shown to confer tolerance to glyphosate, and there is a history of safe use of other EPSPS proteins in commercialized crops (CERA - ILSI Research Foundation, 2011).

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 DAS-Ø1131-3, also referred to as DAS1131 maize.

A.1 (c) Trade name

Maize event DAS1131 is at 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

***Bacillus thuringiensis* (*Bt*): donor of the chimeric *cry*1Da2 gene, which is composed of sequences from *cry*1Da2 and *cry*1Ab**

- Class: Bacillus/Clostridium group (low G+C Gram-positive bacteria)
- Order: Bacillales
- Family: Bacillaceae
- Genus: *Bacillus*
- Species: *B. thuringiensis* (*cry*1Da2); *B. thuringiensis* subsp. *Kurstaki* (*cry*1Ab)
- Strain: PS81I (*cry*1Da2)

Bt is a diverse group of Gram-positive, spore-forming bacteria that has a history of safe use as a pesticide over several decades (US-EPA, 1998; US-EPA, 2001). It occurs ubiquitously in the soil and on plants including vegetables, cotton, tobacco, tree crops, and forest crops (Schnepf et al., 1998; Shelton, 2012). Several Cry proteins have been deployed as safe and effective pest control agents in microbial Bt formulations for almost 40 years. Several Cry proteins have also been effectively deployed as safe and effective pest control agents and have a history of safe use in genetically modified crops (ISAAA, 2019).

***Brassica napus* and *Brassica rapa*: donor of the chimeric chloroplast transit peptide, TraP8**

- Class: Dicotyledoneae (or Magnoliopsida)
- Order: Brassicales (or Cruciales)
- Family: Brassicaceae
- Genus: *Brassica*
- Species: *B. napus* L. and *B. rapa*

B. napus L. and *B. rapa* belongs to the Brassicaceae family, also known as the mustard family. *B. napus* has dark bluish green foliage, branched stems, and yellow flowers (CFIA, 2017). The genus Brassica and its wild relatives are part of the tribe Brassiceae that has its origin in the Mediterranean basin and in south-western Asia (OECD, 2012). Early cultivars of *B. napus*. and *B. rapa* contain high levels of erucic acid and glucosinolates, which are a concern for humans and animal consumption (Eskin and Przybylski, 2003). Varieties lower in erucic acid and glucosinolates have been developed to meet specific standards on the level of erucic acid and glucosinolates (OGTR, 2011).

Streptomyces sviceus: donor of the *dgt-28 epsps* coding sequence

- Class: Actinomycetia (high G+C Gram-positive bacteria)
- Order: Streptomycetales
- Family: Streptomycetaceae
- Genus: *Streptomyces*
- Species: *S. sviceus*

- Strain: ATCC 29083

Streptomyces sviceus is a Gram-positive, aerobic bacterium commonly found in soil. There are very few species within the genus *Streptomyces* that are considered pathogenic to plants (Bignell *et al.*, 2010) or animals (Kämpfer, 2006). *S. sviceus* is not known to be an allergen or toxin. The enzymatic properties of tannase gene from *S. sviceus* is being investigated for application in the food, feed, beverage and pharmaceutical industries (Wu *et al.*, 2015).

A.2 (b) Host organism

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

A.3 Nature of the genetic modification

A.3 (a) Transformation Method

DAS1131 maize was created by *Agrobacterium*-mediated transformation with plasmid PHP88492 (Figure 1; Table 1). Public inbred line B104 (Hallauer *et al.*, 1997) was transformed with plasmid PHP88492 to produce DAS1131 maize. Immature maize embryos were harvested from a surface-sterilized ear of B104 maize approximately 10-14 days after pollination and inoculated with *Agrobacterium tumefaciens* strain DAt13192 (Merlo *et al.*, 2012) containing plasmid PHP88492. *Agrobacterium tumefaciens* strain DAt13192 is a disarmed strain that contains the *vir* genes and enables efficient transfer of the T-DNA region of the transformed plasmid to the inoculated host plant tissue. After 3-4 days of embryo and *Agrobacterium* co‐cultivation on solid culture medium and 7 days of resting on solid culture medium containing the antibiotic carbenicillin to kill *Agrobacterium* without glyphosate herbicide selection, the embryos were transferred to a medium with glyphosate herbicide selection and containing carbenicillin to kill residual *Agrobacterium*. Transformed callus was then transferred to a germination medium and incubated to initiate shoot and root development. Once shoots and roots were established, healthy plants were selected, and PCR was used to confirm the presence of the PHP88492 T-DNA insert. Plants that were regenerated from transformation and tissue culture (designated T0 plants) were selected for further characterization. Refer to Figure 9 for a schematic overview of the transformation and event development process for DAS1131 maize. The subsequent breeding of DAS1131 maize proceeded as indicated in Figure 10 to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial maize lines.

The T-DNA region from plasmid PHP88492 contains two gene cassettes (Figure 2; Table 2). The *cry*1Da2 gene cassette contains a chimeric gene comprised of sequences from the *cry*1Da2 gene encoding an insecticidal core toxin and a derivative of the *cry*1Ab gene, both derived from *Bacillus thuringiensis* (US Patent 9890390 Tan *et al.*, 2018). The expressed Cry1Da2 protein confers control of certain susceptible lepidopteran pests. The Cry1Da2 protein is 603 amino acids in length, inclusive of the last 9 amino acids derived from *cry*1Ab, and has a molecular weight of approximately 68 kDa. Expression of the *cry*1Da2 gene is controlled by the promoter region from the maize ubiquitin gene 1 (*ubi*ZM1), including the 5' untranslated region (UTR) and intron (Christensen *et al.*, 1992). The terminator for the *cry*1Da2 gene is the terminator region from the *ubi*ZM1 gene (Christensen *et al.*, 1992; US Patent 9688996 Kumar *et al.*, 2017).

The *dgt-28 epsps* gene cassette contains a 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) gene derived from *Streptomyces sviceus*, fused to a chimeric chloroplast <u>tra</u>nsit <u>p</u>eptide, TraP8, from *Brassica napus* and *Brassica rapa* (Griffin *et al.*, 2021; WO Patent 2013116700 Lira *et al.*, 2013). The expressed DGT-28 EPSPS protein is targeted to the maize chloroplasts through the TraP8 peptide to provide tolerance to glyphosate herbicide. The deduced expression of the *dgt-28 epsps* gene results in a precursor protein with a total length of 481 amino acids and a molecular weight of approximately 51 kDa which includes the 65-amino acid TraP8 peptide as well as a 2-amino acid linker. Expression of the *dgt-28 epsps* gene is controlled by a second copy of the *ubi*ZM1 promoter, including the 5' UTR and intron, and a second copy of the *ubi*ZM1 terminator.

The PHP88492 T-DNA contains two *att*B recombination sites (*att*B1 and *att*B2) (Hartley *et al.*, 2000; Katzen, 2007), two engineered landing pad regions (ELP1 Region 1 and ELP1 Region 2)

(US Patent 10160975 Ainley *et al.*, 2018), and four zinc finger nuclease target recognition sites (ZFN) (Ainley *et al.*, 2013). The presence of these sites alone does not cause any recombination, since in order to function, these sites need a specific recombinase enzyme that is not naturally present in plants (Cox, 1988; Dale and Ow, 1990; Thorpe and Smith, 1998).

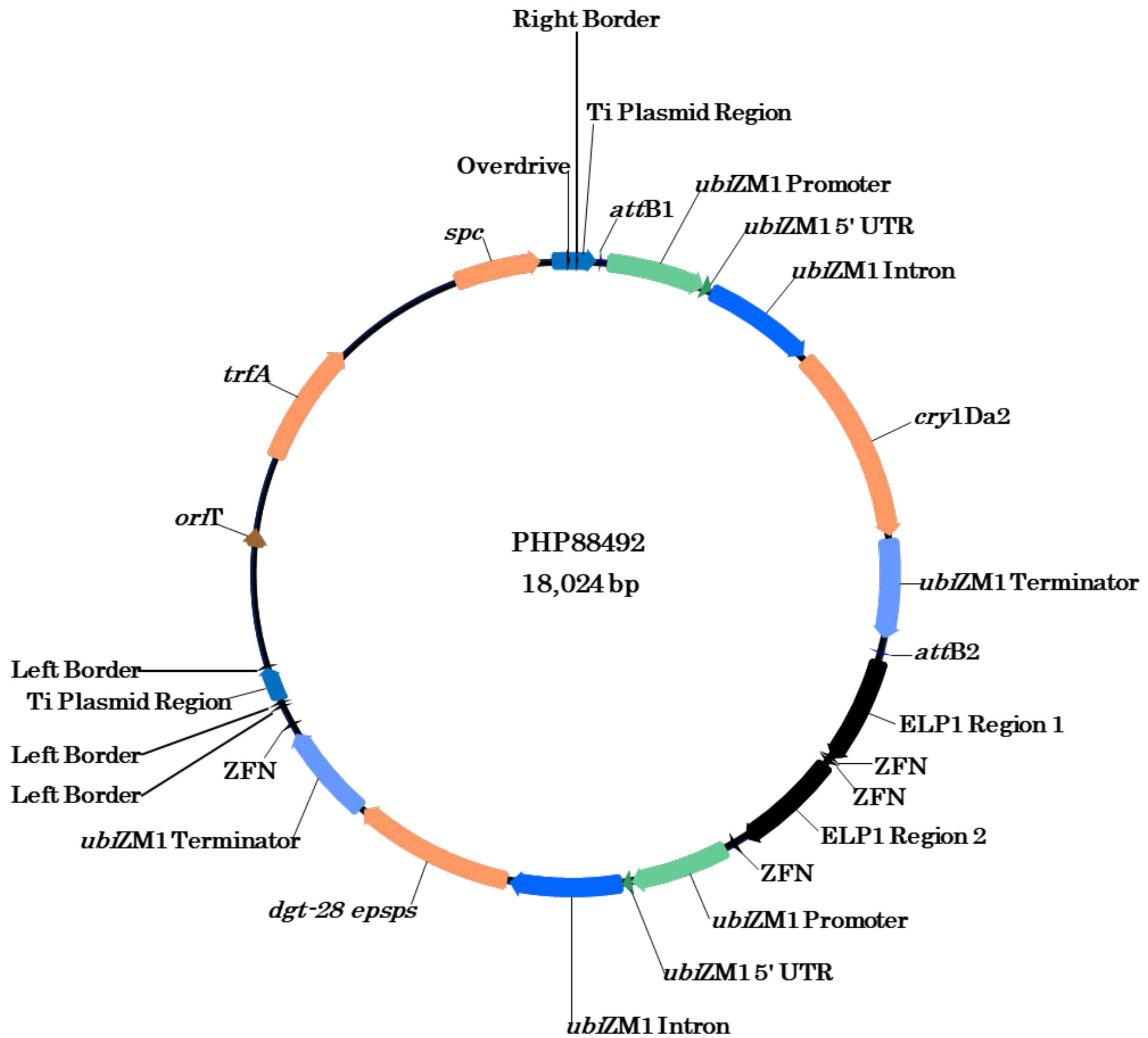


Figure 1. Map of Plasmid PHP88492

Schematic diagram of plasmid PHP88492 indicating the *cry*1Da2 and *dgt-28 epsps* gene cassettes. The size of plasmid PHP88492 is 18,024 bp.

Table 1. Description of the Genetic Elements in Plasmid PHP88492

Region	Location on Plasmid (bp to bp)	Genetic Element	Size (bp)	Description
T-DNA	**1 – 12,664**		**12,664**	**See Table 2 for information on the elements in this region**
Plasmid Construct	**12,665 – 17,877**	**Includes Elements Below**	**5,213**	**DNA from various sources for plasmid construction and plasmid replication**
	13,822 – 13,933	*ori*T	112	Origin of transfer region from bacteria (Komari *et al.*, 1996)
	14,609 – 15,757	*trfA*	1,149	Trans-acting replication gene from bacteria (Komari *et al.*, 1996)
	16,962 – 17,750	*spc*	789	Spectinomycin resistance gene from bacteria (Fling *et al.*, 1985)
Ti Plasmid Backbone	**17,878 – 18,024**	**Includes Elements Below**	**147**	**Overdrive and intergenic regions from the *Agrobacterium tumefaciens* octopine-type Ti plasmid**
	17,878 – 17,986	Ti Plasmid Region	109	Sequence from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)
	17,987 – 18,010	Overdrive	24	T-DNA transmission enhancer from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (Peralta *et al.*, 1986)
	18,011 – 18,024	Ti Plasmid Region	14	Sequence from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)

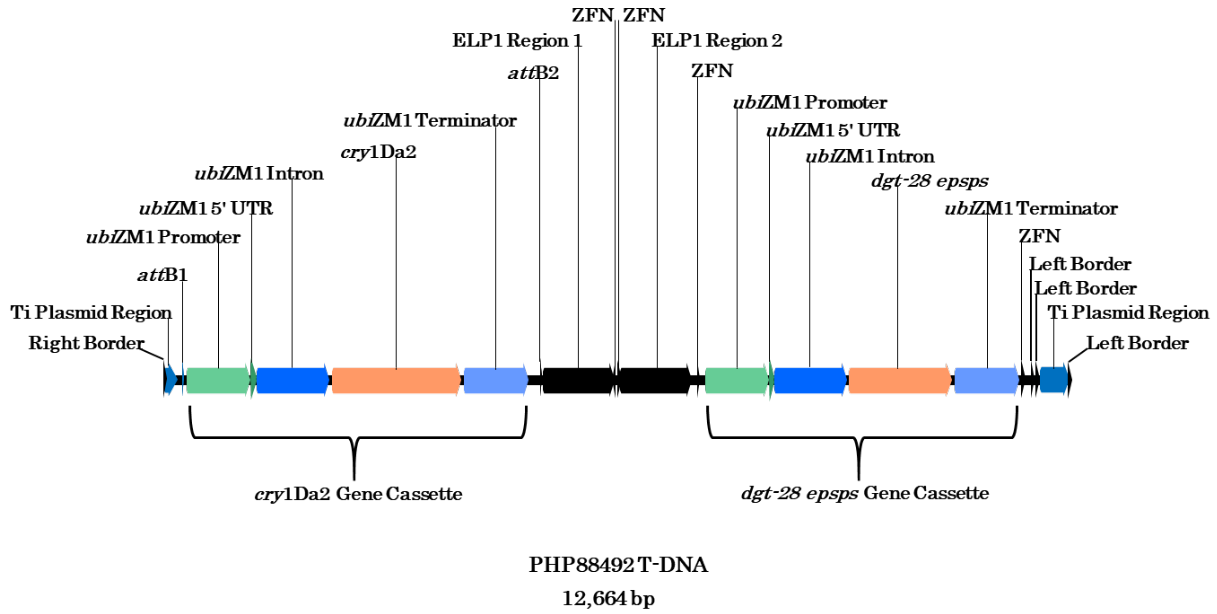


Figure 2. Map of the T-DNA Region from Plasmid PHP88492

Schematic diagram of the PHP88492 T-DNA region indicating the *cry*1Da2 and *dgt-28 epsps* gene cassettes. The size of the T-DNA is 12,664 bp.

Table 2. Description of the Genetic Elements in the T-DNA Region from Plasmid PHP88492

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	1 – 24	Right Border (RB)	24	T-DNA Right Border from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)
	25 – 132	Ti plasmid Region	108	Sequence from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)
	133 – 247	Intervening Sequence	115	DNA sequence used for cloning
	248 – 271	*att*B1	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning system (Hartley *et al.*, 2000; Katzen, 2007)
	272 – 316	Intervening Sequence	45	DNA sequence used for cloning
*cry*1Da2 gene cassette	317 – 1,211	*ubi*ZM1 Promoter	895	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	1,212 – 1,294	*ubi*ZM1 5' UTR	83	5' untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	1,295 – 2,310	*ubi*ZM1 Intron	1,016	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	2,311 – 2,349	Intervening Sequence	39	DNA sequence used for cloning
	2,350 – 4,161	*cry*1Da2	1,812	Chimeric gene comprised of sequences from the *cry*1Da2 gene encoding an insecticidal core toxin and a derivative of the *cry*1Ab gene, both derived from *Bacillus thuringiensis* (US Patent 9890390 Tan *et al.*, 2018) as described below: *cry*1Da2 at bp 2,350 - 4,131 (1,782 bp long) *cry*1Ab at bp 4,132 - 4,161 (30 bp long)
	4,162 – 4,194	Intervening Sequence	33	DNA sequence used for cloning
	4,195 – 5,104	*ubi*ZM1 Terminator	910	Terminator region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992; US Patent 9688996 Kumar *et al.*, 2017)
	5,105 – 5,269	Intervening Sequence	165	DNA sequence used for cloning
	5,270 – 5,293	*att*B2	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning System (Hartley *et al.*, 2000; Katzen, 2007)
	5,294 – 5,310	Intervening Sequence	17	DNA sequence used for cloning
	5,311 – 6,310	ELP1 Region 1	1,000	Engineered landing (US Patent 10160975 Ainley *et al.*, 2018)

Table 2. Description of Genetic Elements in the T-DNA Region from Plasmid PHP88492 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	6,311 – 6,344 (complementary)	ZFN	34	Zinc finger nuclease target site (Ainley *et al.*, 2013)
	6,345 – 6,378	ZFN	34	Zinc finger nuclease target site (Ainley *et al.*, 2013)
	6,379 – 7,378	ELP1 Region 2	1,000	Engineered landing pad (US Patent 10160975 Ainley *et al.*, 2018)
	7,379 – 7,476	Intervening Sequence	98	DNA sequence used for cloning
	7,477 – 7,513	ZFN	37	Zinc finger nuclease target site (Ainley *et al.*, 2013)
	7,514 – 7,580	Intervening Sequence	67	DNA sequence used for cloning
dgt-28 epsps gene cassette	7,581 – 8,475	*ubi*ZM1 Promoter	895	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	8,476 – 8,558	*ubi*ZM1 5' UTR	83	5' untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	8,559 – 9,574	*ubi*ZM1 Intron	1,016	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen *et al.*, 1992)
	9,575 – 9,613	Intervening Sequence	39	DNA sequence used for cloning
	9,614 – 11,059	*dgt-28 epsps*	1,446	5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) gene derived from *Streptomyces sviceus*, fused to a chimeric chloroplast transit peptide, TraP8, from *Brassica napus* (WO Patent 2013116700 Lira *et al.*, 2013) and *Brassica rapa* (Griffin *et al.*, 2021) as described below: TraP8 at bp 9,614 - 9,808 (195 bp long) Peptide linker at bp 9,809 - 9,814 (6 bp long) *dgt-28 epsps* at bp 9,815 - 11,059 (1,245 bp long)
	11,060 – 11,085	Intervening Sequence	26	DNA sequence used for cloning
	11,086 – 11,995	*ubi*ZM1 Terminator	910	Terminator region from the *Zea mays* gene ubiquitin gene 1 (Christensen *et al.*, 1992; US Patent 9688996 Kumar *et al.*, 2017)

Table 2. Description of Genetic Elements in the T-DNA Region from Plasmid PHP88492 (continued)

Gene Cassette	Location on T-DNA (bp to bp)	Genetic Element	Size (bp)	Description
	11,996 – 12,064	Intervening Sequence	69	DNA sequence used for cloning
	12,065 – 12,101	ZFN	37	Zinc finger nuclease target site (Ainley *et al.*, 2013)
	12,102 – 12,285	Intervening Sequence	184	DNA sequence used for cloning
	12,286 – 12,309	Left Border	24[1]	T-DNA Left Border from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)
	12,310 – 12,328	Intervening Sequence	19	DNA sequence used for cloning
	12,329 – 12,353	Left Border	25	T-DNA Left Border from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)
	12,354 – 12,376	Intervening Sequence	23	DNA sequence used for cloning
	12,377 – 12,639	Ti Plasmid Region	263	Sequence from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)
	12,640 – 12,664	Left Border	25	T-DNA Left Border from the *Agrobacterium tumefaciens* octopine-type Ti plasmid (GenBank accession AF242881.1; Komari *et al.*, 1996)

[1] Shortened by 1 bp on the 3' end

A.3 (b) Description of the construct and the transformation vectors used

Please refer to Section A.3(a) *Transformation method* for the vectors used in transformation and to Table 1 for the description of the genetic elements in Plasmid PHP88492, Figure 1 for the map of Plasmid PHP88492, Table 2 for the description of the genetic elements in the T-DNA region from Plasmid PHP88492 and Figure 2 for the map of the T-DNA region from Plasmid PHP88492.

A.3 (c) Molecular characterisation

Characterization of the inserted DNA in DAS1131 maize was conducted using a Next Generation Sequencing (NGS) method known as Southern-by-Sequencing (SbS™ technology, hereafter referred to as SbS) to determine the insertion copy number and organisation within the plant genome and to confirm the absence of plasmid backbone sequences. Sanger sequencing was conducted to determine the DNA sequence of the DAS1131 insert and flanking genomic regions. Based on the determined genomic border sequences, nucleotide (BLASTN) searches were performed to identify the genomic location of the insert and to determine if any endogenous genes were disrupted by the insert. Additionally, a bioinformatic safety assessment of translated stop-

codon-bracketed reading frames within an insertion or crossing the boundary between an insertion and its genomic borders was conducted to evaluate for similarity to known and putative allergens and toxins. An event-specific quantitative real-time PCR method was developed and validated for detection of event DAS-Ø1131-3 in maize.

Southern-by-Sequencing (SbS) Analysis to Determine Insertion Copy Number and Organization and Confirm the Absence of Plasmid Backbone Sequences (PHI-2021-044 study)

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. By compiling a large number of unique sequencing reads and mapping them against the transformation plasmid and control maize genome, unique junctions due to inserted DNA are identified in the bioinformatics analysis and used to determine the insertion copy number and organization within the plant genome and confirm the absence of plasmid backbone 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 sequence reads where part of the read shows exact homology to the plasmid DNA sequence while the rest of the read does not match the contiguous plasmid. Junctions may occur between inserted DNA and genomic DNA, or between insertions of two plasmid-derived DNA sequences that are not contiguous in the transformation plasmid. Multiple sequence reads are generated for each junction and are compiled into a consensus sequence for the junction. By compiling a large number of unique sequencing reads and comparing them to the transformation plasmid and control maize genome, unique junctions due to inserted DNA are identified. A unique junction is defined as one in which the adjacent sequences are the same across multiple reads, although the overall length of the multiple reads for that junction will vary due to the sequencing process. The number of unique junctions is related to the number of plasmid insertions present in the maize genome (for example, a single T-DNA insertion is expected to have two unique junctions). Detection of additional unique junctions beyond the two expected for a single insertion would indicate the presence of rearrangements or additional insertions derived from plasmid DNA. The absence of any junctions indicates there are no detectable insertions within the maize genome.

The T1 generation of DAS1131 maize was analyzed by SbS, using capture probes targeting all sequences of plasmid PHP88492, to determine the insertion copy number and organization and to confirm the absence of plasmid backbone sequences. SbS was also performed on one B104 control maize plant, and on a positive control sample containing PHP88492 plasmid DNA to confirm that the assay could reliably detect plasmid DNA spiked into control maize genomic DNA at a level equivalent to one copy of plasmid per genome copy. Based on the results obtained for DAS1131 maize, a schematic diagram of the DAS1131 insertion was developed and is provided in Figure 4.

Several genetic elements in plasmid PHP88492 are derived from maize, and thus the homologous elements in the B104 maize genome will be captured by the full-coverage probes used in the SbS analysis. These endogenous elements (*ubi*ZM1 promoter, 5 UTR, intron, and terminator; Table 3

and Figure 1 and Figure 2) will have sequencing reads in the SbS results due to the homologous elements in the B104 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 4 and the positive control sample is presented in Figure 5. Results from the segregating T1 generation of DAS1131 maize are presented in Figure 6, Figure 7 and Appendix A - Figures A1 to A5.

SbS Analysis of the B104 Control Maize

Sequencing reads of the B104 control maize were aligned to the PHP88492 T-DNA and plasmid maps (Figure 4); 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 B104 control maize genome (Table 3). Variation in coverage of the maize endogenous elements is due to sequence variations between the B104 control maize and the maize varieties from which the genetic elements in the plasmid were derived. No junctions were detected between plasmid sequences and the maize genome (Table 4), 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 B104 control maize genome.

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

SbS analysis of the positive control sample resulted in sequence coverage across the entire length of plasmid PHP88492 (Figure 5), indicating that the SbS assay utilizing the full-coverage probe library is sensitive enough to detect PHP88492 sequences at a concentration equivalent to one copy of plasmid per copy of the maize genome. No junctions were detected between plasmid and genomic sequences (Table 4), indicating that the sequence reads were due to either the spiked-in plasmid or the endogenous maize genetic elements that were detected in the B104 control maize.

SbS Analysis of the T1 Generation of DAS1131 Maize

SbS analysis of ten plants of the segregating T1 generation of DAS1131 maize showed six positive plants that contained the inserted DNA (Table 4, Figure 6; and Appendix A - Figures A1 to A5). Each of these plants contained two unique genome-insertion junctions, one at each end of the insertion, that were identical across the six plants. The insertion, derived from the PHP88492 T-DNA, starts with the 5′ junction at bp 28 and ends with the 3′ junction at bp 12,274 (Figure 4). The number of sequencing reads at the 5′ and 3′ junctions is provided in Table 4. There were no other junctions between the PHP88492 plasmid and the maize genome detected in the plants, indicating that there are no additional plasmid-derived insertions present in DAS1131 maize. 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 backbone sequence of PHP88492 and maize genome sequences, demonstrating that no plasmid backbone sequences were incorporated into DAS1131 maize.

Each of the four DAS1131 maize plants from the T1 generation that was determined to be negative for the DAS1131 insertion yielded sequencing reads for the endogenous genetic elements derived from the maize genome (a representative plant is presented in Figure 7). 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 PHP88492.

SbS analysis of the T1 generation of DAS1131 maize demonstrated that DAS1131 maize contains a single copy of the inserted DNA derived from the PHP88492 T-DNA, and that no additional insertions or plasmid backbone sequences are present in its genome.

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

Table 3. Maize Endogenous Elements in Plasmid PHP88492 and DAS1131 Insertion

Number[a]	Name of Endogenous Element[b]	Present in Plasmid or Insertion
1	*ubi*ZM1 promoter, 5′ UTR, and intron	PHP88492, DAS1131 insertion
2	*ubi*ZM1 terminator	PHP88492, DAS1131 insertion

[a] The numbers indicating endogenous genetic elements are shown as circled numbers found below the linear maps in Figure 4 and Figure 5 and Appendix A Figures A1-A5.

[b] As shown in the plasmid and T-DNA maps in Figure 1 and Figure 2, respectively.

Table 4. SbS Junction Reads

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]	DAS1131 Insertion
T1 Generation Plant ID 404421221	0	0	0	0	-
T1 Generation Plant ID 404421222	1342	173	976	148	+
T1 Generation Plant ID 404421223	1111	157	758	135	+
T1 Generation Plant ID 404421224	1405	180	1042	158	+
T1 Generation Plant ID 404421225	1310	172	793	109	+
T1 Generation Plant ID 404421226	0	0	0	0	-
T1 Generation Plant ID 404421227	0	0	0	0	-
T1 Generation Plant ID 404421228	1316	192	1084	189	+
T1 Generation Plant ID 404421229	0	0	0	0	-
T1 Generation Plant ID 404421230	1994	252	1760	263	+
B104 Control Maize	0	0	0	0	-
PHP88492 Positive Control	0	0	0	0	-

[a] Total number of sequence reads across the 5′ junction of the DAS1131 insertion.

[b] Unique sequence reads establishing the location of the 5′ genomic junction of the DAS1131 insert (Figure 4). Multiple identical NGS-supporting reads are condensed into each unique read.

[c] Total number of sequence reads across the 3′ junction of the DAS1131 insertion.

[d] Unique sequence reads establishing the location of the 3′ genomic junction of the DAS1131 insert (Figure 4). Multiple identical NGS-supporting reads are condensed into each unique read.

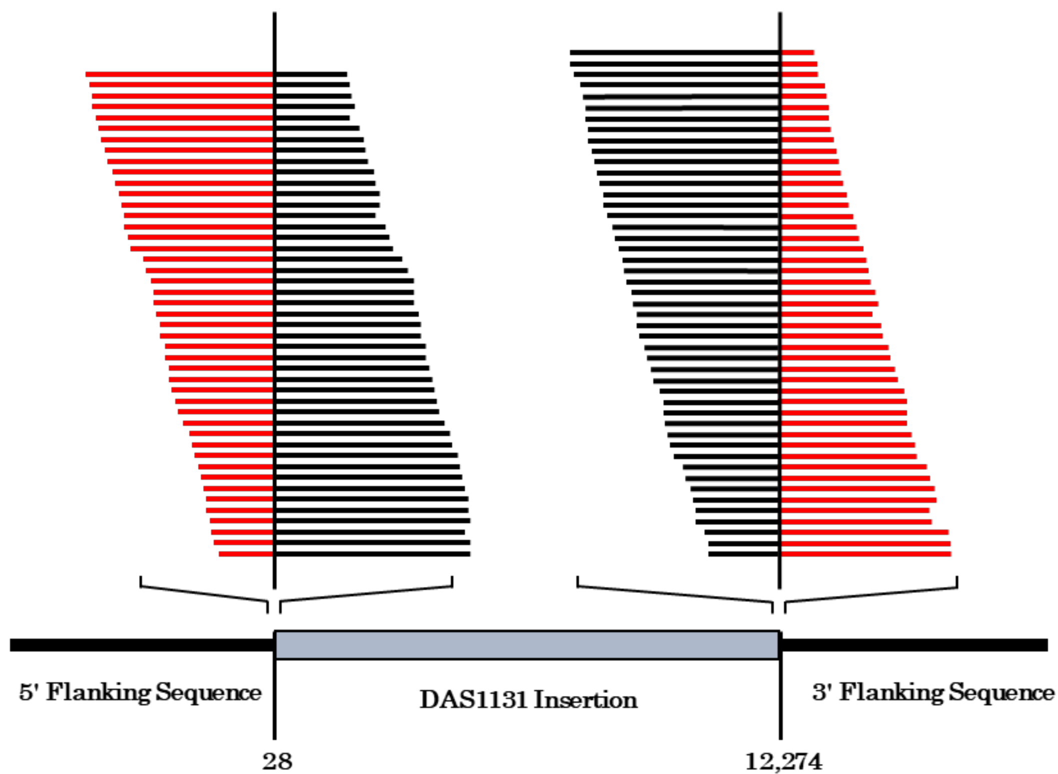
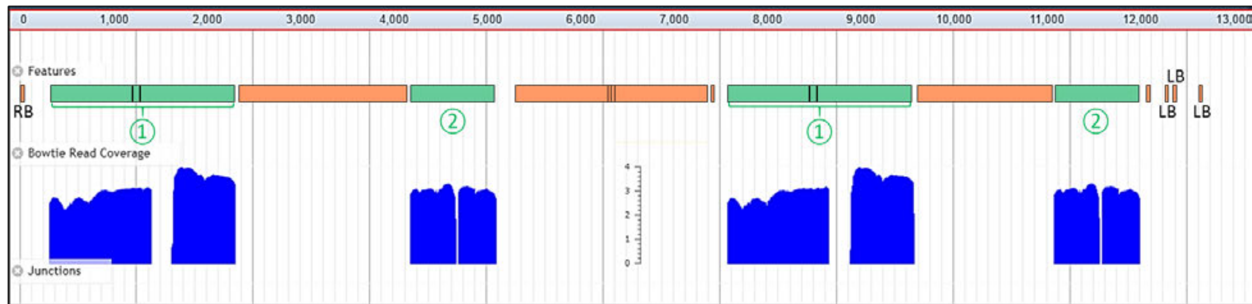


Figure 3. Schematic Diagram of the DAS1131 Maize DNA Insertion

Schematic diagram of the DNA insertion in DAS1131 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 the PHP88492 T-DNA and shown by the gray box, is integrated into the DAS1131 maize genome. Vertical lines show the locations of the two unique genome-plasmid junctions. The numbers below the map indicate the bp location of the junction nucleotide in reference to the sequence of the PHP88492 T-DNA (Figure 2). Representative individual sequencing reads across the junctions are shown as horizontally stacked lines above each junction (not to scale); red indicates genomic flanking sequence and black indicates inserted DNA sequence within each read.

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

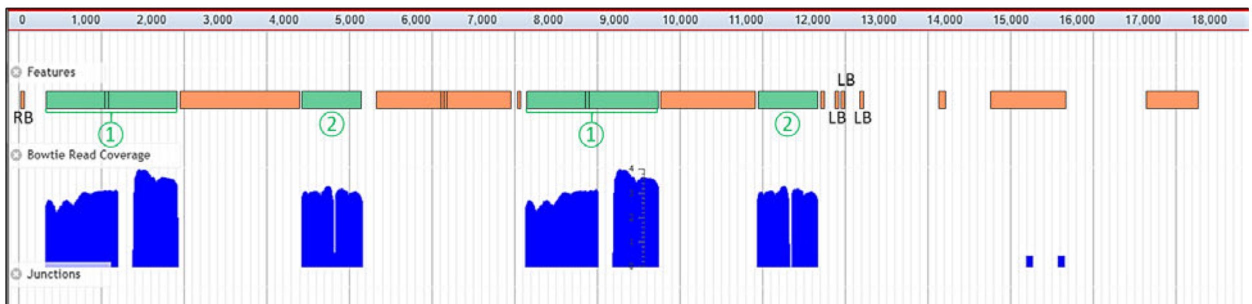


Figure 4. SbS Results for Control Maize

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 3), 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 B104 control maize. A) SbS results for B104 control maize aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2). 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 B104 control maize, and the sequence reads are solely due to the endogenous elements present in the B104 genome. B) SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 1). Coverage was obtained only for the endogenous elements.

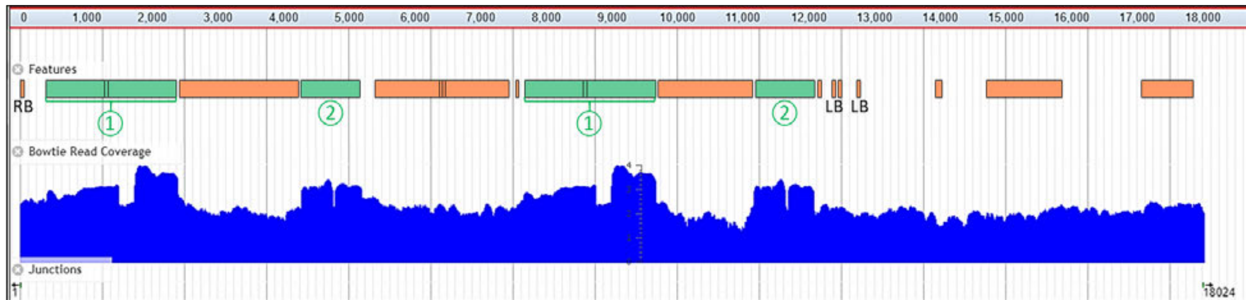
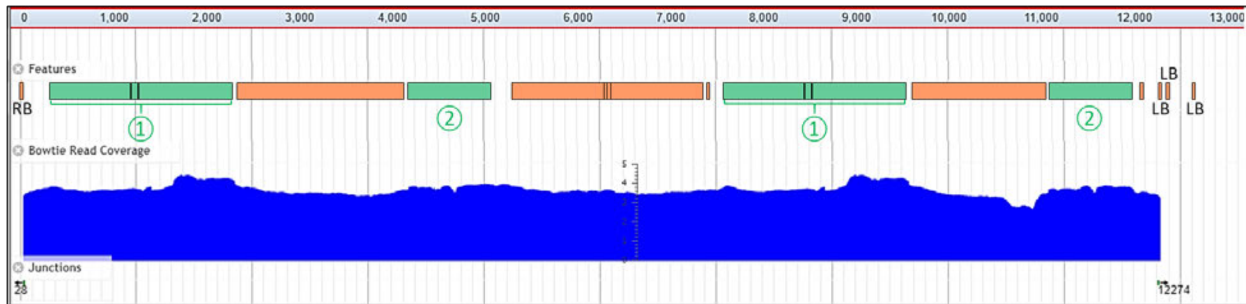


Figure 5. SbS Results for PHP88492 Positive Control Sample

The positive control sample consisted of the B104 control maize DNA spiked with plasmid PHP88492 at a level corresponding to one copy of plasmid per copy of the maize genome. Shown are the SbS results of the positive control sample aligned against PHP88492 (18,024 bp; Figure 1). 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 3), while tan bars indicate genetic elements derived from other sources. Junctions shown at the bottom of the graph 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. Coverage was obtained across the full length of the plasmid, indicating successful capture of PHP88492 sequences by the SbS probe library.

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

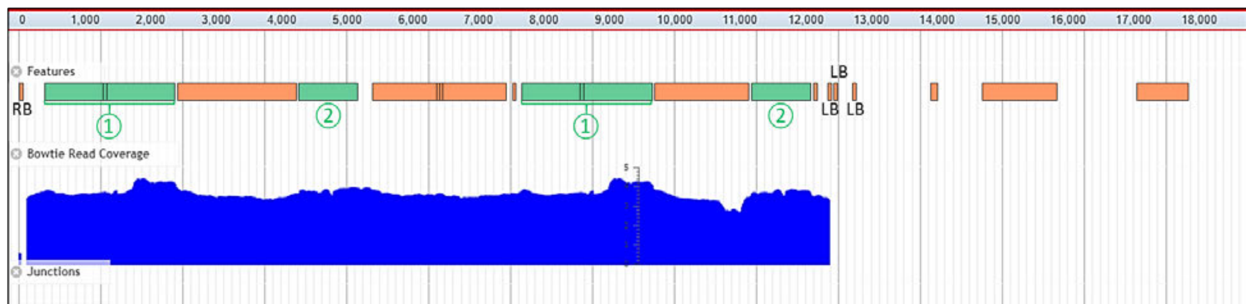
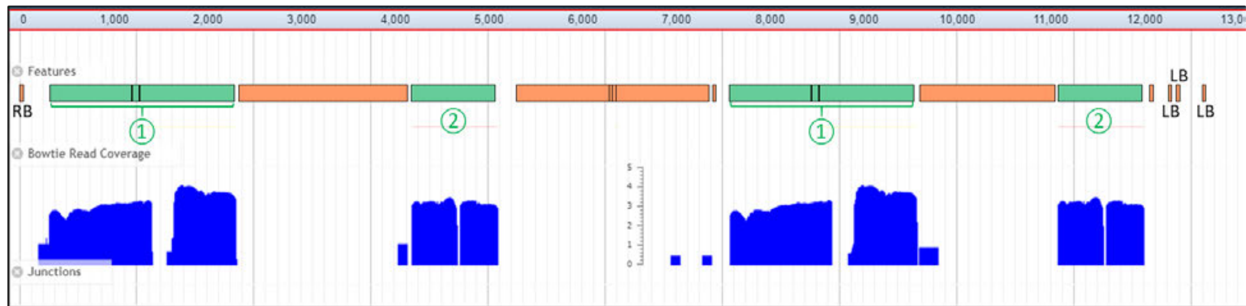


Figure 6. SbS Results for DAS113 Maize (Plant ID 404421230)

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 3), while tan bars indicate genetic elements derived from other sources. A) SbS results aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2) indicating that this plant contains the insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers below the arrows refer to the bp location of the junction relative to the PHP88492 T-DNA (Figure 1). The insertion comprises bp 28 to 12,274 of the PHP88492 T-DNA shown in Figure 2. The presence of only two junctions demonstrates the presence of a single insertion in the DAS1131 maize genome. B) SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 1). Coverage was obtained for the elements between the Right and Left Borders transferred into DAS1131 maize (region between the RB and LB elements); however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP88492 sequence shows that there are no additional insertions or plasmid backbone sequences present in DAS1131 maize.

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

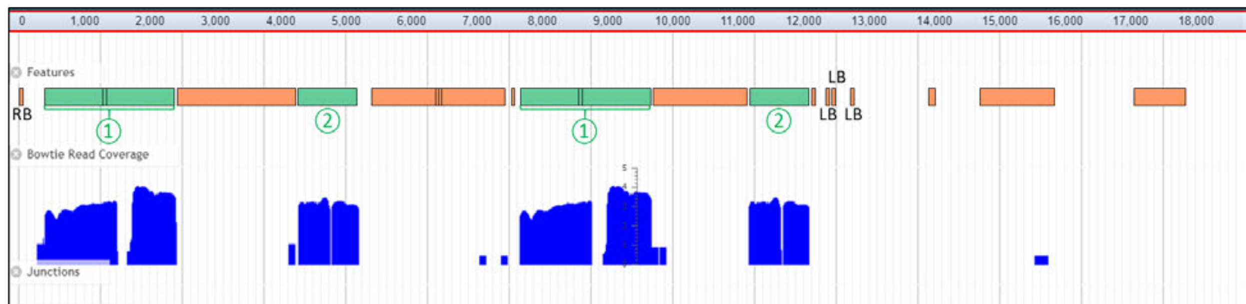


Figure 7. SbS Results for Representative Null Segregant Plant (Plant ID 404421227)

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 3), while tan bars indicate genetic elements derived from other sources. A) SbS results aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2), indicating that this plant does not contain the insertion. 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 this plant, and the sequence reads are solely due to the endogenous elements present in the B104 genome. B) SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 1). Coverage was obtained only for the endogenous elements. The absence of any junctions between plasmid and genomic sequences indicates that there are no insertions or plasmid backbone sequences present in this plant from the T1 generation of DAS1131 maize.

Sequence of the Insert and Genomic Border Regions (PHI-2020-173 study)

Sequence characterization analysis was performed to determine the DNA sequence of the DAS1131 insert and flanking genomic regions. It should be noted that while DNA sequencing provides certain molecular information, the exact nucleotide sequence should not be viewed as static. Spontaneous mutations are a very common phenomenon in plants, presenting a biological mechanism of adaptation to constantly changing environment (Weber et al., 2012). Spontaneous mutations can occur in any part of the plant genome and in both non-GM and GM plants (Waigmann et al., 2013). In GM plants, there is no scientific basis to expect that the frequency of spontaneous mutations in transgenic insert or flanking genomic regions would be greater than in the rest of the plant genome, or that they would have a differential impact on safety (La Paz et al., 2010; Waigmann et al., 2013).

The total length of sequence determined in DAS1131 maize is 14,969 bp, comprised of 1,289 bp of the 5’ flanking genomic sequence, 1,433 bp of the 3’ flanking genomic sequence, and 12,247 bp of inserted DNA from the PHP88492 T-DNA (Figure 8). The inserted T-DNA in the DAS1131 maize analysed was confirmed to have a 27-bp deletion at the right border end and a 390-bp deletion at the left border end, and a single A-to-G change at bp 1,954 in the *ubi*ZM1 promoter. Right Border and Left Border termini deletions often occur in *Agrobacterium*-mediated transformation (Kim *et al.*, 2007). All remaining sequence is intact and identical to that of the PHP88492 T-DNA.

Additional details regarding analytical methods for Sanger sequencing analysis are provided in Appendix D.

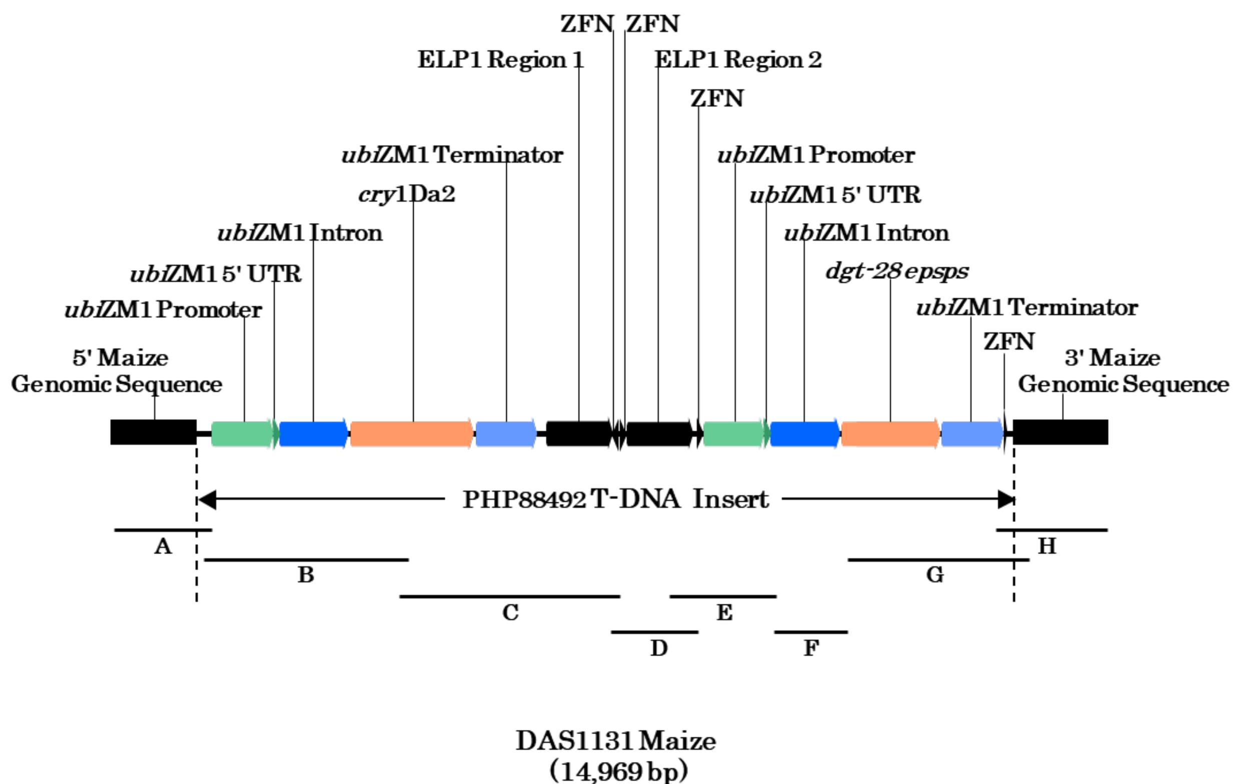


Figure 8. Map of the Insert and Flanking Genomic Regions Sequenced in DAS1131 Maize

Eight overlapping PCR fragments (**A**, **B**, **C**, **D**, **E**, **F**, **G**, and **H**) covering the inserted DNA and flanking genomic regions were amplified from genomic DNA of DAS1131 maize. Each black horizontal bar represents the relative position of the PCR fragment, and the vertical dash lines represent the genomic border and insert junctions. The total length of confirmed sequence for the DAS1131 insert and flanking genomic regions is 14,969 bp, comprised of 1,289 bp of the 5' flanking genomic sequence, 1,433 bp of the 3' flanking genomic sequence, and 12,247 bp of inserted DNA from the PHP88492 T-DNA.

Flanking Border Analysis (PHI-2021-171/230 study)

A bioinformatic assessment of the genomic border flanking the DAS1131 maize insertion was conducted to determine the chromosomal location of the insertion and confirm there was no disruption of endogenous genes or regulatory elements. It should be noted that while bioinformatic assessment of the border flanking an insertion in a GM plant provides some molecular information, genes and regulatory elements may be disrupted or deleted in a plant without any safety risks. For example, genes are disrupted or deleted in conventionally bred plants, and it is the high plasticity of plant genomes that enables selective breeding for desired traits. Conventional breeding is generally regarded as safe, and the likelihood and risks of a gene disruption or deletion occurring in a GM plant is the same as in a conventionally bred plant (Herman et al., 2011). Flanking border analysis confirmed the chromosomal location of the insertion and confirmed no known genes or regulatory elements were disrupted or deleted.

Detailed methods and results are provided in the PHI-2021-171/230 study.

Translated Stop Codon-bracketed Reading Frame Analysis of the Insert/Border Junctions (PHI-2021-173/225 study)

Assessing translated stop codon-bracketed reading 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 reading frames was conducted following established international criteria (Codex Alimentarius Commission, 2003; EFSA, 2010; EFSA, 2011; FAO/WHO, 2001). All translated stop codon-bracketed reading frames of length ≥ eight amino acids in maize (*Zea mays* L.) event DAS-Ø1131-3 (referred to as DAS1131 maize) that are within the insertion or that cross the boundary between the insertion and its genomic borders were identified and evaluated.

A total of 723 translated stop codon-bracketed reading frames ≥ eight amino acids were identified for the DAS1131 maize sequence. Among them, 195 are open-reading frames (ORFs) starting with methionine, including the ORFs encoding for the Cry1Da2 and DGT-28 EPSPS proteins.

The allergen database used for the searches was the Comprehensive Protein Allergen Resource (COMPARE) 2021 database (January 2021), 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 2348 sequences.

Six of the translated stop codon-bracketed reading frames ≥ 29 aa in DAS1131 maize returned alignments with percent identity > 35% and alignment length ≥ 80 aa from the search against the allergen database. However, further analysis indicated that these alignments are likely false positive. None of the translated stop codon-bracketed reading frames in DAS1131 maize produced an eight contiguous amino acid match to an allergen. These data indicate that there is no allergenicity concern regarding the translated stop codon-bracketed reading frames in DAS1131 maize.

The potential toxicity of the translated stop codon-bracketed reading frames was assessed by comparison of their sequences to the sequences in an internal toxin database. The internal toxin

database is a subset of sequences found in UniProtKB/Swiss-Prot. To produce the internal toxin database, the proteins in UniProtKB/Swiss-Prot are filtered for molecular function by keywords that could imply toxicity or adverse health effects (e.g., toxin, hemagglutinin, vasoactive, etc.). The internal toxin database is updated annually. The searches between the translated stop codon-bracketed reading frames and protein sequences in the internal toxin database was conducted with BLASTP using default parameters, except that all the alignments at or below the *E*-value threshold were returned. Alignments returned between a translated stop codon-bracketed reading frame and an internal toxin database protein sequence with an *E*-value $\leq 10^{-4}$ were examined more closely.

To further characterize the translated stop codon-bracketed reading frames, additional searches were conducted against the extensive, publicly-available National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database (NCBI-nr). The searches between the translated stop codon-bracketed reading frames and protein sequences in the NCBI-nr protein database were conducted with BLASTP using default parameters. Alignments returned between a translated stop codon-bracketed reading frame and an NCBI-nr protein sequence with an *E*-value $\leq 10^{-4}$ were examined more closely.

No alignments with an *E*-value $\leq 10^{-4}$ were returned between a translated stop codon-bracketed reading frame and any protein sequence in the internal toxin database. Eleven translated stop codon-bracketed reading frames produced alignments to protein sequences in the NCBI-nr protein database with *E*-values $\leq 10^{-4}$, none to toxins. None of these proteins are toxic to human and animals.

In conclusion, bioinformatics evaluation of the DAS1131 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.

Detailed methods and results are provided in the PHI-2021-173/225 study.

Event-Specific Detection Methodology (PHI-2021-042 study)

An event-specific quantitative real-time PCR method was developed and validated for detection of event DAS-Ø1131-3 in maize. The event-specific assay for DAS1131 maize is designed to amplify the target sequence at the 5' junction between the DAS1131 maize insertion and the maize genomic DNA. The binding site of the forward primer is within the maize genomic DNA; the binding site of the reverse primer is within the DAS1131 maize insert and the binding site of the probe spans the junction of the transgenic insertion and the maize genomic DNA. The event-specific PCR assay for DAS1131 maize amplifies a 98-bp product.

Conclusions on the Characterization of the Inserted DNA in DAS1131 Maize

SbS, Sanger sequencing of the insert and genomic border regions, and bioinformatic analyses of the border sequences and the translated stop codon-bracketed reading frames were conducted to characterize the inserted DNA in DAS1131 maize.

SbS analysis confirmed that DAS1131 maize contains a single copy of the inserted DNA with the expected organisation, and that no additional insertions or plasmid backbone sequences are present in DAS1131 maize. The total length of sequence determined by Sanger sequencing for DAS1131

maize is 14,969 bp, comprised of 1,289 bp of the 5' flanking genomic sequence, 1,433 bp of the 3' flanking genomic sequence, and 12,247 bp of inserted DNA from the PHP88492 T-DNA (Figure 8). The inserted T-DNA in the DAS1131 maize analysed was confirmed to have a 27-bp deletion at the right border end and a 390-bp deletion at the left border end, and a single A-to-G change at bp 1,954 in the first copy of the *ubi*ZM1 promoter. All remaining sequence is intact and identical to that of the PHP88492 T-DNA. A bioinformatic assessment of the genomic border flanking the DAS1131 maize insertion confirmed the chromosomal location of the insertion and confirmed there was no disruption of endogenous genes. A bioinformatics evaluation of the DAS1131 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.

Together, these analyses confirmed that a single copy of the inserted DNA with no plasmid backbone sequences is present in the DAS1131 maize genome. Bioinformatic analyses support the conclusion that there is unlikely to be allergenicity or toxicity concerns regarding the putative translated frames at the DAS1131 insertion site. Additionally, an event-specific quantitative real-time PCR detection method was developed and validated for DAS1131 maize.

A.3 (d) Breeding process

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 DAS1131 maize is provided in Figure 9.

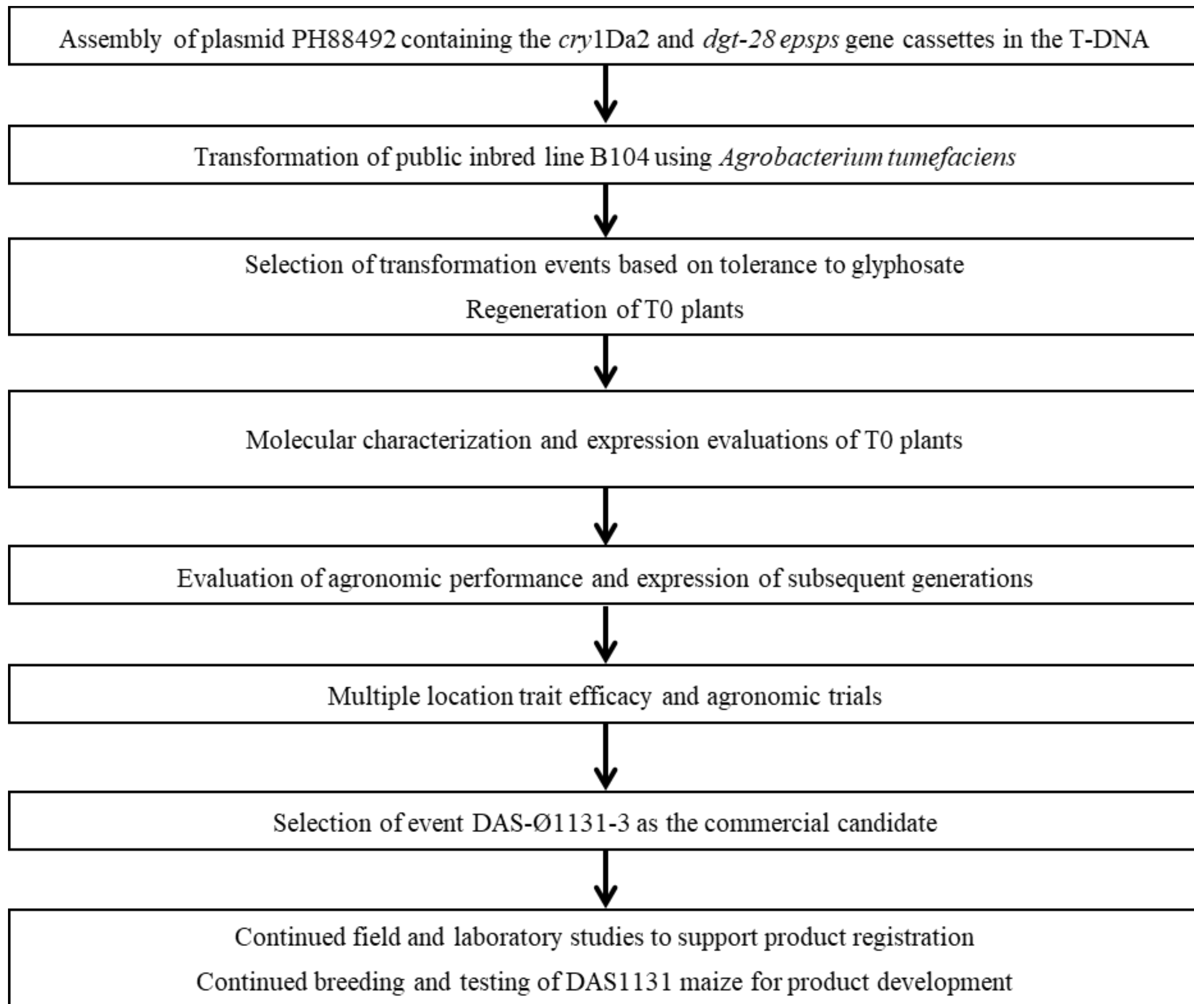


Figure 9. Event Development Process of DAS1131 Maize

The subsequent breeding of DAS1131 maize proceeded as indicated in Figure 10 to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial maize lines. Table 5 provides the generations used for each characterization study.

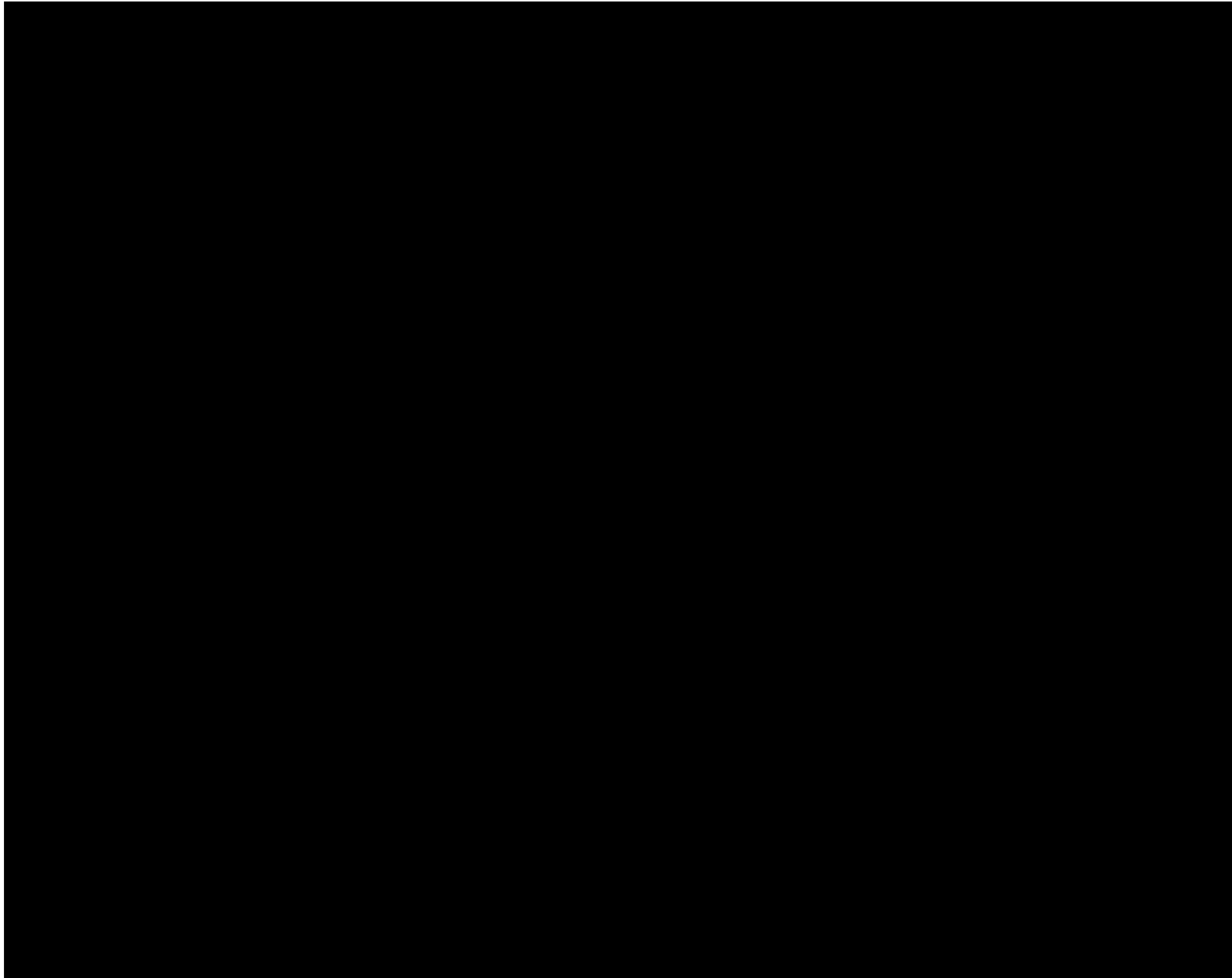


Figure 10. Breeding Diagram for DAS1131 Maize and Generations Used for Analysis

The breeding steps to produce the generations used for characterization, assessment, and the development of commercial lines are shown schematically. Public inbred line B104 was used for transformation to produce DAS1131 maize.

Table 5. Generations and Comparators Used for Analysis of DAS1131 Maize

Analysis	Seed Generation(s) Used	Comparators
Insertion copy number, insertion organization, and the absence of plasmid backbone sequences by SbS	T1	B104
Insertion organization and stability by Southern blot	T1, T2, T3, T4, and T6	B104
Sanger sequencing	T3	B104
Mendelian inheritance by multi-generation segregation analysis	(BC1F1 [B104/PH184C], BC1F1 [B104/PH1V5T], T2, T4, and T6)	N/A
Composition and expression analysis	F1 (B104/PH4257)	B104/PH4257

Selection of Comparators

For the characterization of DAS1131 maize, the inbred line B104 and the F1 hybrid B104/PH4257 were used as experimental controls (Table 5). The control lines selected are non-genetically modified (non-GM) and represent the same genetic background of the maize lines used to produce the DAS1131 maize generations used in analysis (Figure 10).

In addition, conventionally bred (conventional) non-GM maize hybrid lines (i.e., reference lines), were used to obtain tolerance intervals for compositional analyses. These maize hybrids were chosen to represent a wide range of conventional non-GM commercial varieties. These tolerance intervals help represent the range of biological variation of maize for compositional analytes and further helped to determine the comparability of DAS1131 maize to conventional non-GM maize.

A.3 (e) Stability of the genetic changes

Southern Analysis to Determine Stable Genetic Inheritance across Generations (PHI-2021-051 study)

Southern blot analysis was performed on five generations of DAS1131 maize to evaluate the stability of the inserted *cry*1Da2 and *dgt-28 epsps* gene cassettes across multiple generations.

Restriction enzymes [redacted] and [redacted] were selected to verify the stability of the DAS1131 maize insertion across the five generations (T1, T2, T3, T4, and T6) of DAS1131 maize plants. [redacted] was selected because there is a single [redacted] restriction site within the DAS1131 maize insertion (indicated in Figure 11 and Figure 12), which provides a means to uniquely identify the event, as additional sites would be in the adjacent flanking genomic DNA (Figure 13). Due to its large recognition site sequence, [redacted] is not expected to cut often in the maize genome; therefore, would yield large fragments that may not separate well by agarose gel electrophoresis. [redacted] does not have a recognition site within the DAS1131 insertion and was added to [redacted] to reduce the size of the digested DNA fragments and allow improved separation of the Southern blot bands.

Genomic DNA samples isolated from leaf tissues of the five generations of DAS1131 maize and the B104 control maize were double digested with [redacted] and [redacted] and hybridized with the *cry*1Da2 and *dgt-28 epsps* probes for Southern analysis. Hybridization patterns of these probes exhibited event-specific bands unique to the DAS1131 maize insertion, and thus provided a means of verification that the genomic border regions of the DAS1131 maize insertion were not changed across the five generations during breeding. Plasmid PHP88492 was added to control maize DNA, digested with [redacted] and [redacted] and included on the blot to verify successful probe hybridization. The probes used for Southern hybridization are described in Table 6 and shown in Figure 12.

Hybridization of the *cry*1Da2 probe to [redacted]-digested genomic DNA resulted in a consistent band of approximately [redacted] in all five generations of DAS1131 maize samples analyzed (Table 7, Figure 14). This result confirmed that the 5' border fragment, containing the *cry*1Da2 gene in the DAS1131 maize insertion, is intact and stable across the five generations of DAS1131 maize. The lanes containing PHP88492 plasmid DNA showed the expected band of 18,024 bp, confirming successful hybridization of the *cry*1Da2 probe.

Hybridization of the *dgt-28 epsps* probe to [redacted]-digested genomic DNA resulted in a single band of approximately [redacted] in all five generations of DAS1131 maize analyzed (Table 7, Figure 15). This result confirmed that the 3' border fragment, containing the *dgt-28 epsps* gene

in the DAS1131 maize insertion, is intact and stable across the five generations of DA1131 maize. The lanes containing PHP88492 plasmid DNA showed the expected band of 18,024 bp, confirming successful hybridization of the *dgt-28 epsps* probe.

The presence of equivalent bands from hybridization with the *cry*1Da2 and *dgt-28 epsps* probes within the plants from all five generations analyzed confirms that the inserted DNA in DAS1131 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 6. Description of DNA Probes Used for Southern Hybridization

Genetic Element/ Probe Name	Probe Length (bp)	Position on PHP88492 T-DNA (bp to bp)[a]
*cry*1Da2[b]		
dgt-28 epsps[c]		

[a] The probe position is based on the PHP88492 T-DNA map (Figure 12).
[b] The *cry*1Da2 probe comprises two fragments that are combined in a single hybridization solution.
[c] The *dgt-28 epsps* probe comprises two fragments that are combined in a single hybridization solution.

Table 7. Predicted and Observed Hybridization Bands on Southern Blots; Digest

Probe Name	Predicted and Observed Fragment Size from Plasmid PHP88492 (bp)[a]	Predicted Fragment Size from PHP88492 T-DNA (bp)[b]	Observed Fragment Size in DAS1131 Maize[c] (bp)	Figure
*cry*1Da2	18,024			Figure 14
dgt-28 epsps	18,024			Figure 15

[a] Predicted and observed fragment sizes are based on the PHP88492 plasmid map (Figure 11).
[b] Predicted sizes are based on the PHP88492 T-DNA (Figure 12).
[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.

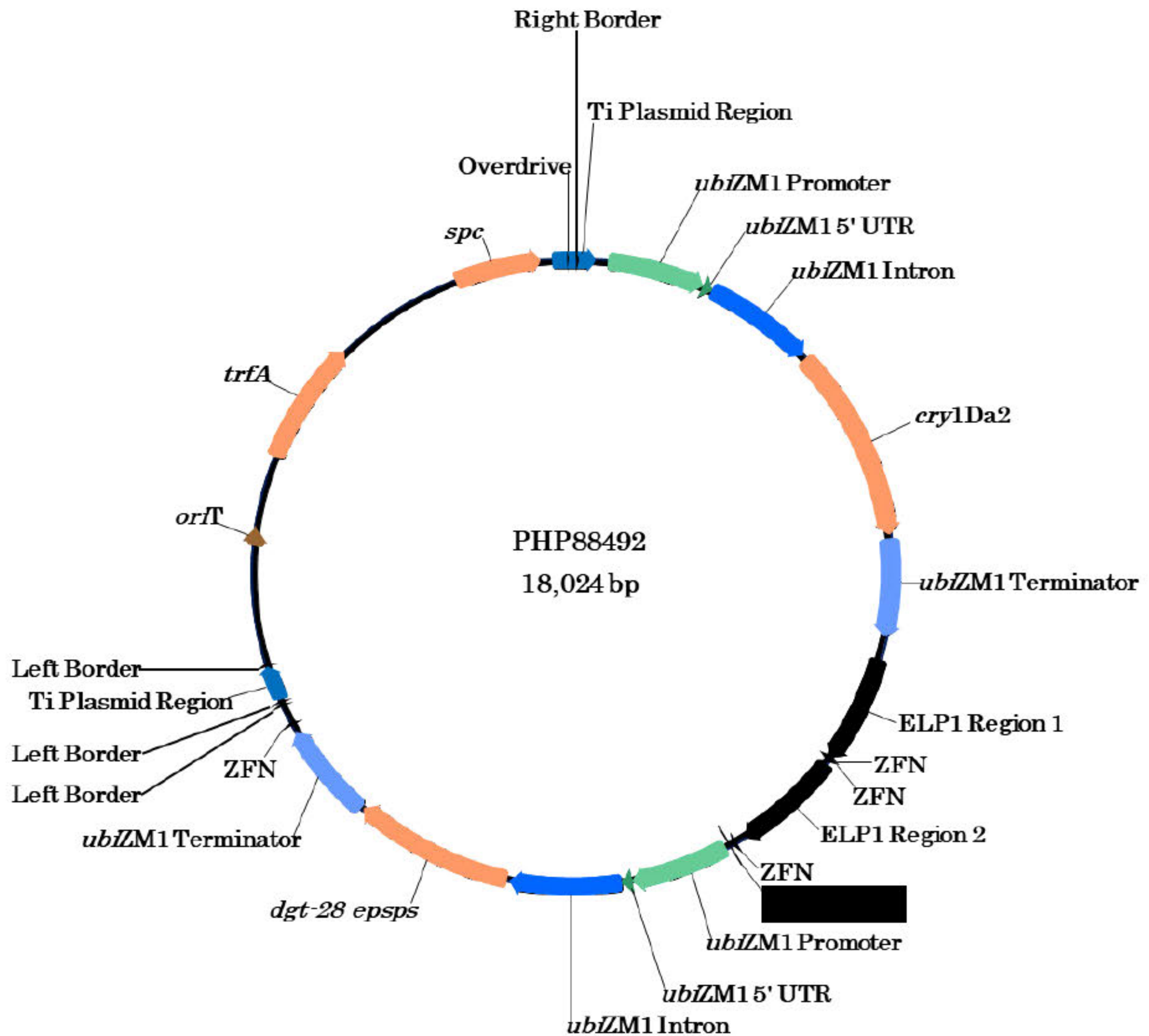
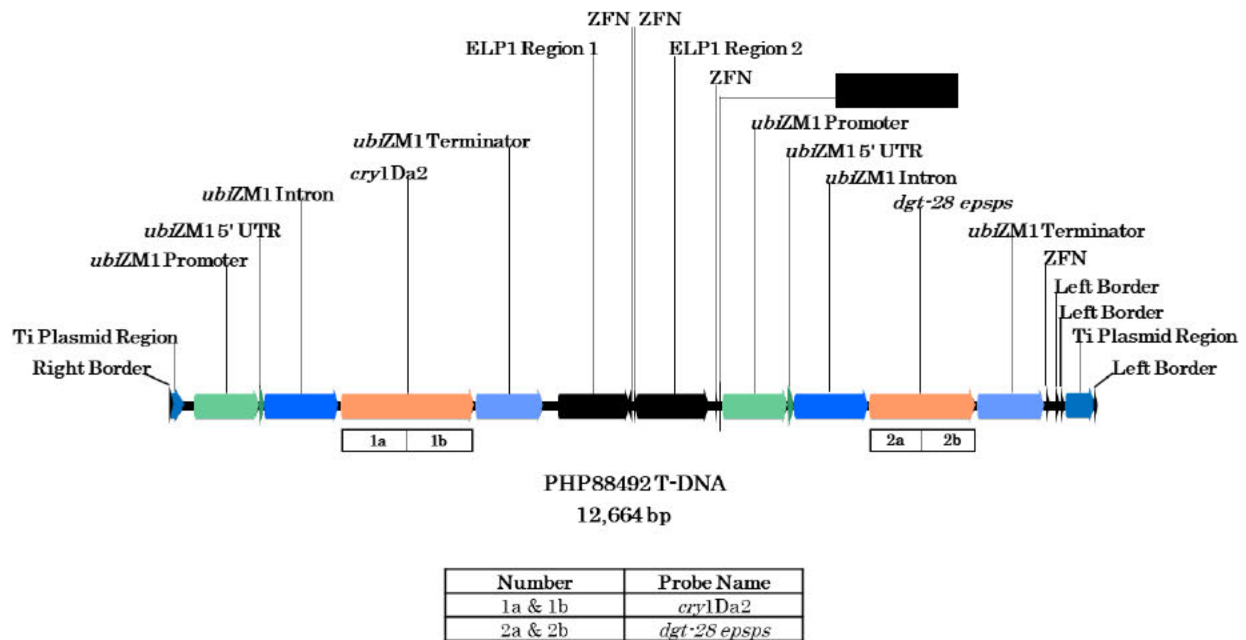


Figure 11. Map of Plasmid PHP88492 for Southern Analysis

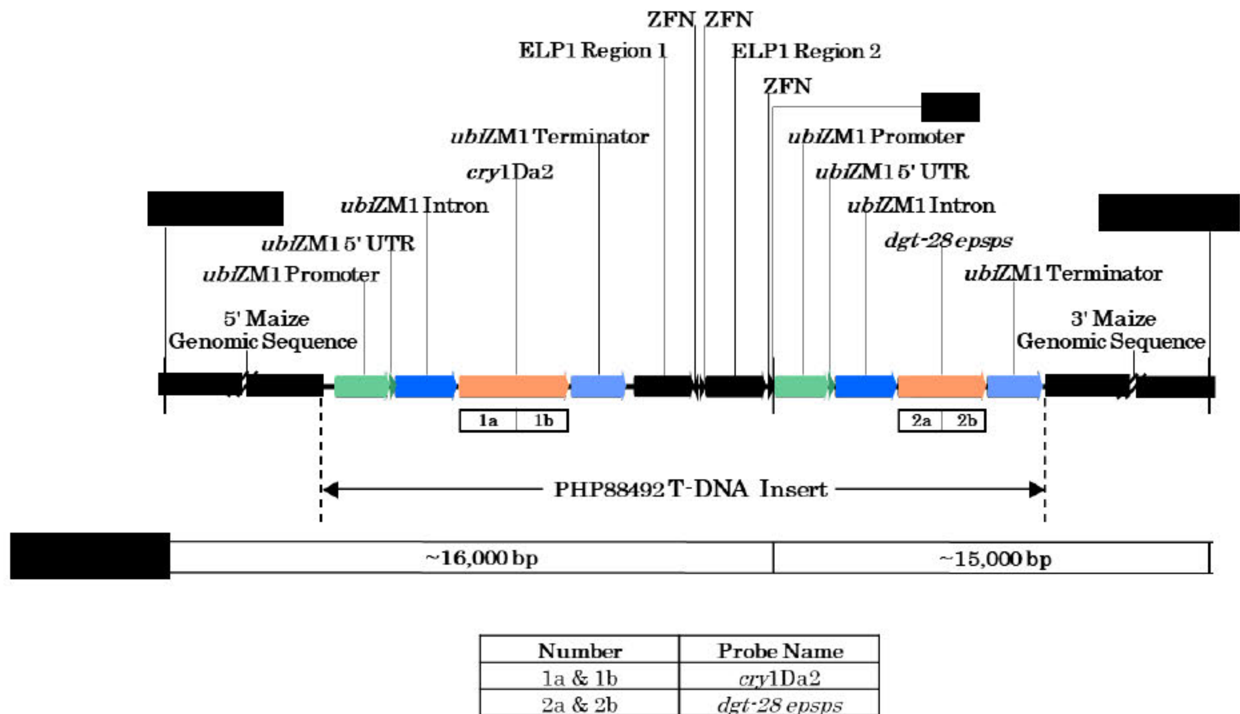
Schematic diagram of plasmid PHP88492 indicating the [redacted] restriction enzyme site with base pair position. The size of plasmid PHP88492 is 18,024 bp. The Right Border and Left Border flank the T-DNA (Figure 12) that was transferred during *Agrobacterium*-mediated transformation to create DAS1131 maize. There are no *Afl* II sites in PHP88492.



Number	Probe Name
1a & 1b	*cry*1Da2
2a & 2b	*dgt-28 epsps*

Figure 12. Map of the PHP88492 T-DNA for Southern Analysis

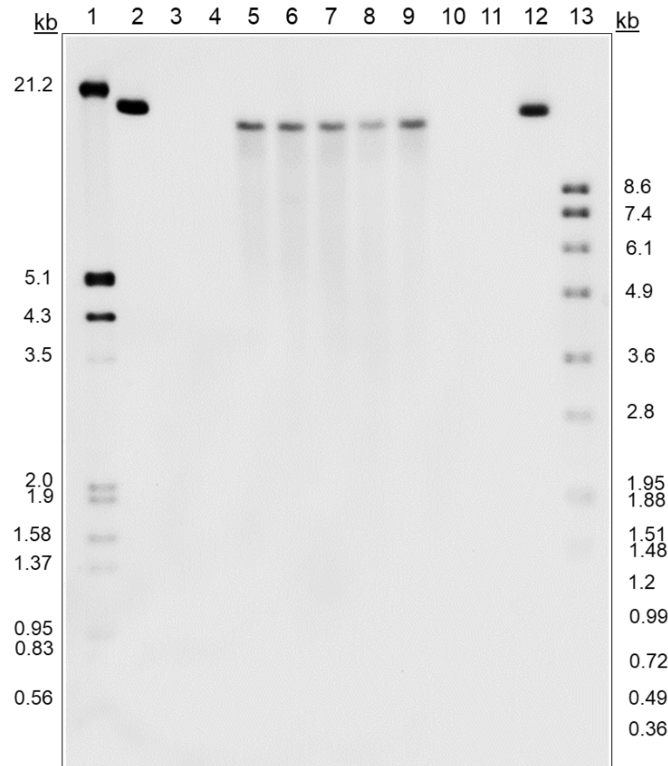
Schematic diagram of the PHP88492 T-DNA indicating the [redacted] restriction enzyme site with base pair position. The size of the PHP88492 T-DNA is 12,664 bp. The locations of the Southern blot probes are shown by the boxes below the map. There are no [redacted] sites in the PHP88492 T-DNA.



Number	Probe Name
1a & 1b	*cry*1Da2
2a & 2b	*dgt-28 epsps*

Figure 13. Map of the DAS1131 Insertion

Schematic diagram of the DAS1131 maize insertion indicating the [redacted] and [redacted] restriction enzyme sites. The locations of the Southern blot probes are shown by the boxes below the map. The flanking maize genomic sequences are represented by the horizontal black rectangular bars. The [redacted] and [redacted] 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.

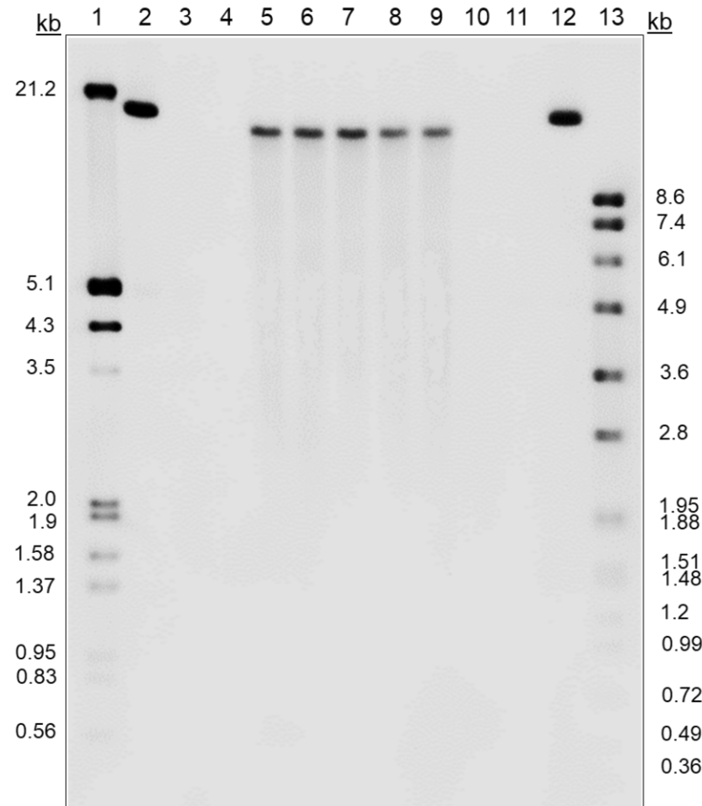


Lane	Sample
1	DIG-labeled DNA marker III
2	1 copy of PHP88492 + B104 control maize
3	B104 Control maize
4	Blank
5	DAS1131 maize T1 generation
6	DAS1131 maize T2 generation
7	DAS1131 maize T3 generation

Lane	Sample
8	DAS1131 maize T4 generation
9	DAS1131 maize T6 generation
10	Blank
11	B104 Control maize
12	1 copy of PHP88492 + B104 control maize
13	DIG-labeled DNA marker VII

Figure 14. Southern Blot Analysis of DAS1131 Maize; [redacted] Digest with cry1Da2 Probe

Genomic DNA isolated from leaf tissues of DAS1131 maize from T1, T2, T3, T4, and T6 generations and B104 control maize plants, was digested with [redacted] and hybridized to the *cry*1Da2 probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP88492 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 PHP88492 + B104 control maize
3	B104 Control maize
4	Blank
5	DAS1131 maize T1 generation
6	DAS1131 maize T2 generation
7	DAS1131 maize T3 generation

Lane	Sample
8	DAS1131 maize T4 generation
9	DAS1131 maize T6 generation
10	Blank
11	B104 Control maize
12	1 copy of PHP88492 + B104 control maize
13	DIG-labeled DNA marker VII

Figure 15. Southern Blot Analysis of of DAS1131 Maize; ████ ████ █ Digest with *dgt-28 epsps* Probe

Genomic DNA isolated from leaf tissues of DAS1131 maize from T1, T2, T3, T4, and T6 generations and B104 control maize plants, was digested with ████████ and hybridized to the *dgt-28 epsps* probe. Approximately 10 µg of genomic DNA was digested and loaded per lane. Positive control lanes include PHP88492 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).

Multi-Generation Segregation Analysis (PHI-2020-068 study)

Segregation analysis was performed on five generations of DAS1131 maize 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 DAS1131 maize (BC1F1 [B104/PH184C], BC1F1 [B104/PH1V5T], T2, T4, and T6 generations) were analyzed using genotypic and phenotypic analyses. The selected maize generations represent a range of different genotypes, created through crossing, backcrossing, and selfing, in a typical maize breeding program.

The genotypic analyses utilized a quantitative polymerase chain reaction (qPCR) assay on each individual plant to confirm the copy number of event DAS-Ø1131-3 and the *cry*1Da2 and *dgt-28 epsps* genes. The phenotypic analysis utilized a visual herbicide injury evaluation to confirm the presence or absence of tolerance to glyphosate for each individual plant.

The individual results for each plant were compared to the qPCR results to verify co-segregation of genotype with phenotype. All plants were identified as positive or negative with positive plants tolerant to glyphosate and confirmed to contain all the transgenes. A chi-square statistical test (at the 0.05 significance level) was conducted for the qPCR results of the segregating generations of DAS1131 maize to compare the observed segregation ratios to the expected segregation ratios (1:1 for the BC1F1 generation (entries 1 and 2) and 3:1 for the T2 generation). A chi-square test was not performed for the T4 and T6 generations of DAS1131 maize as all plants were identified as positive (i.e., not segregating) as expected for a homozygous generation.

A summary of segregation results for DAS1131 maize is provided in Table 8. For each individual plant in all generations, all genotypic results matched the corresponding phenotypic result, indicating the DNA insertion co-segregated with the trait phenotype and was stable through traditional breeding. No statistically significant differences were found between the observed and expected segregation ratios for each of the three segregating generations of DAS1131 maize.

The results of the multi-generation segregation analysis demonstrated that the inserted DNA in DAS1131 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 during the breeding process.

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

Table 8. Summary of Genotypic and Phenotypic Segregation Analyses for Five Generations of DAS1131 Maize

Entry	Generation	Expected Segregation Ratio	Observed Segregation[a]			Statistical Analysis	
		(Positive:Negative)	Positive	Negative	Total	Chi-Square[b]	P-Value
1	BC1F1	1:1	52	48	100	0.16	0.6892
2	BC1F1	1:1	53	47	100	0.36	0.5485
3	T2	3:1	70	30	100	1.33	0.2482
4	T4	Homozygous	100	0	100	--	--
5	T6	Homozygous	100	0	100	--	--

[a] The observed segregation ratio was determined by combining genotype and phenotype results.
[b] Degrees of freedom = 1. A chi-square value greater than 3.84 (P-value less than 0.05) would indicate a significant difference.

B. CHARACTERISATION AND SAFETY ASSESSMENT OF NEW SUBSTANCES

B.1 Characterisation and safety assessment of new substances

There are no new substances associated with DAS1131 maize other than the two proteins (see Section B.2 *New proteins* below).

B.2 New proteins

Cry1Da2 protein

Amino Acid Sequence of the Cry1Da2 Protein

The Cry1Da2 protein is encoded by the *cry*1Da2 gene, a chimeric gene comprised of sequences from the *cry*1Da2 gene encoding an insecticidal core toxin and a derivative of the *cry*1Ab gene, both derived from *Bacillus thuringiensis*. The deduced amino acid sequence shown below is from the translation of the *cry*1Da2 gene. The full-length protein is 603 amino acids in length and has a molecular weight of approximately 68 kDa (Figure 16).

```
  1   MEINNQNQCV PYNCLSNPKE IILGEERLET GNTVADISLG LINFLYSNFV
 51   PGGGFIVGLL ELIWGFIGPS QWDIFLAQIE QLISQRIEEF ARNQAISRLE
101   GLSNLYKVYV RAFSDWEKDP TNPALREEMR IQFNDMNSAL ITAIPLFRVQ
151   NYEVALLSVY VQAANLHLSI LRDVSVFGER WGYDTATINN RYSDLTSLIH
201   VYTNHCVDTY NQGLRRLEGR FLSDWIVYNR FRRQLTISVL DIVAFFPNYD
251   IRTYPIQTAT QLTREVYLDL PFINENLSPA ASYPTFSAAE SAIIRSPHLV
301   DFLNSFTIYT DSLARYAYWG GHLVNSFRTG TTTNLIRSPL YGREGNTERP
351   VTITASPSVP IFRTLSYITG LDNSNPVAGI EGVEFQNTIS RSIYRKSGPI
401   DSFSELPPQD ASVSPAIGYS HRLCHATFLE RISGPRIAGT VFSWTHRSAS
451   PTNEVSPSRI TQIPWVKAHT LASGASVIKG PGFTGGDILT RNSMGELGTL
501   RVTFTGRLPQ SYYIRFRYAS VANRSGTFRY SQPPSYGISF PKTMDAGEPL
551   TSRSFAHTTL FTPITFSRAQ EEFDLYIQSG VYIDRIEFIP VTATLEAESD
601   LER*
```

Figure 16. Deduced Amino Acid Sequence of the Cry1Da2 Protein

The deduced amino acid sequence from the translation of the *cry*1Da2 gene from plasmid PHP88492. The asterisk (*) indicates the translational stop codon. The full-length protein is 603 amino acids in length, inclusive of the last 9 amino acids derived from *cry*1Ab, and has a molecular weight of approximately 68 kDa.

Function and Activity of the *cry*1Da2 Protein

The Cry1Da2 protein is encoded by the *cry*1Da2 gene, a chimeric gene comprised of sequences from the *cry*1Da2 gene encoding an insecticidal core toxin and a derivative of the *cry*1Ab gene, both derived from *Bacillus thuringiensis*. The expressed Cry1Da2 protein binds to receptors in the brush border membrane of susceptible lepidopteran pests and causes cell death through the

formation of non-specific, ion conducting pores in the apical membrane of the midgut epithelial cells.

Characterization of the Cry1Da2 Protein Derived from DAS1131 Maize and Microbial System (PHI-2021-148, PHI-2020-028 and PHI-2020-235 studies)

The Cry1Da2 protein expressed in DAS1131 maize was purified from the V9 leaf tissue using immuno-affinity chromatography.

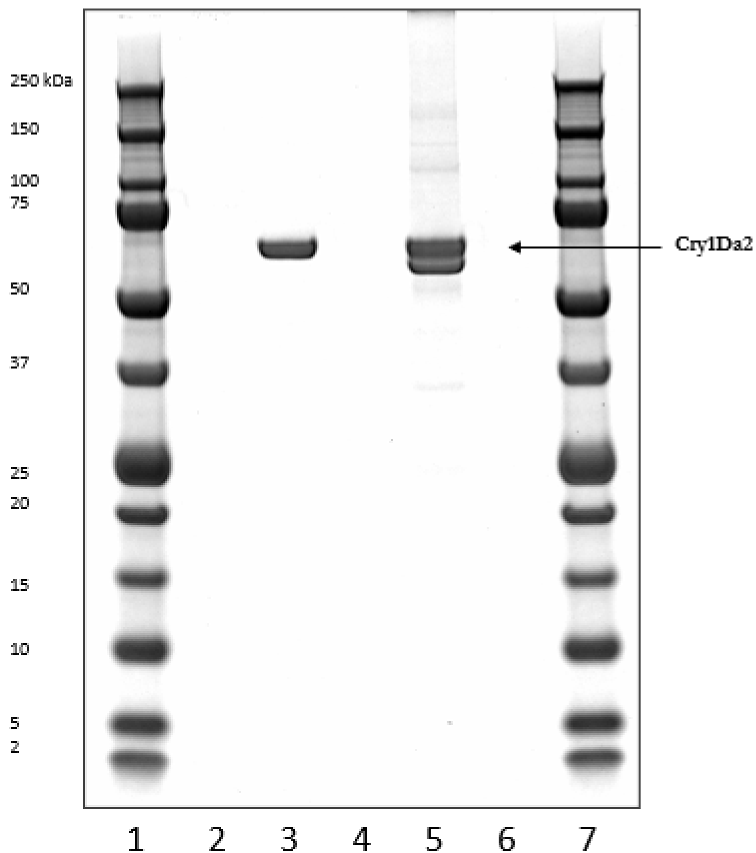
In order to have sufficient amounts of purified Cry1Da2 protein for the multiple studies required to assess its safety, the Cry1Da2 protein was expressed in a [redacted] protein expression system through fermentation. The microbially derived protein was purified from the cell lysate using inclusion body washing techniques and anion exchange chromatography. The inclusion bodies were solubilized and then digested with trypsin. Following trypsin digestion, the Cry1Da2 protein was purified using anion exchange chromatography prior to diafiltration to concentrate the protein and exchange the buffer into 10 mM CAPS, pH 11.0. The microbially derived Cry1Da2 protein contains two intended amino acid modifications relative to the DAS1131 maize-derived protein. Both the lysine at position 19 and the arginine at position 27 were changed to glutamine to avoid truncation and ensure intactness of the N-terminus during production of the test substance.

The biochemical characteristics of the microbially derived Cry1Da2 protein and the DAS1131 maize-derived Cry1Da2 protein were characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, protein glycosylation analysis, peptide mapping by liquid chromatography mass spectrometry (LC-MS), and N terminal amino acid sequencing. For the microbially derived Cry1Da2 protein, the bioactivity was verified by insect bioassays. The results demonstrated that the DAS1131 maized-derived and microbially derived Cry1Da2 proteins are of the expected molecular weight, immunoreactivity, lack of glysoylation, and amino acid sequence. The microbially derived Cry1Da2 protein was demonstrated to be an appropriate test substance for use in safety studies.

SDS-PAGE Analysis

Samples of the DAS1131 maize-derived Cry1Da2 protein and the microbially derived Cry1Da2 protein were analyzed by SDS-PAGE. The SDS-PAGE gel demonstrated the microbially derived Cry1Da2 protein migrated as a predominant band consistent with the expected molecular weight of approximately 68 kilodaltons (kDa) (Figure 17). The DAS1131 maize-derived Cry1Da2 protein migrated as two predominant bands: the upper Cry1Da2 protein band was consistent with the expected molecular weight of approximately 68 kDa and the molecular weight observed for the microbially derived Cry1Da2 protein. The lower band migrated at approximately 66 kDa (Figure 17; also see Peptide Mapping section and Table 10 for additional information about the N-terminal truncation).

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	Microbially Derived Cry1Da2 Protein (Lot # PRCH-006)[b]
4	1X LDS/DTT Sample Buffer Blank
5	DAS1131 Maize-Derived Cry1Da2 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 ofthe predicted molecular weight.

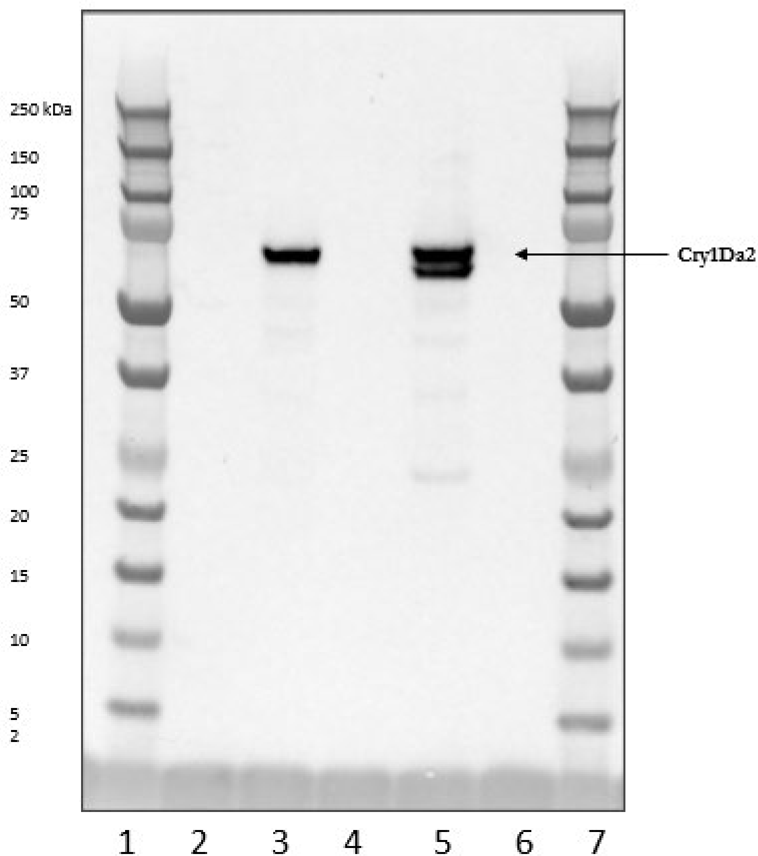
[b] Target concentration of 1 µg.

Figure 17. SDS-PAGE Analysis of Cry1Da2 Protein

Western Blot Analysis

Samples of the DAS1131 maize-derived Cry1Da2 protein and the microbially derived Cry1Da2 protein were analyzed by Western blot. Western blot analysis demonstrated that the microbially derived Cry1Da2 protein and the DAS1131 maize-derived Cry1Da2 protein were immunoreactive to a Cry1Da2 polyclonal antibody (Figure 18). The microbially derived Cry1Da2 protein was visible as a predominant band consistent with the expected molecular weight of approximately 68 kDa. The DAS1131 maize-derived Cry1Da2 protein was visible as two predominant bands as the same molecular weights identified during SDS-PAGE analysis: the upper band with the expected molecular weight of approximately 68 kDa and the lower band with a molecular weight of approximately 66 kDa (Figure 17; also see Peptide Mapping section and Table 10 for additional information about the N-terminal truncation).

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	Microbially Derived Cry1Da2 Protein (Lot # PRCH-006)[b]
4	1X LDS/DTT Sample Buffer Blank
5	DAS1131 Maize-Derived Cry1Da2 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 concentration of 10 ng.

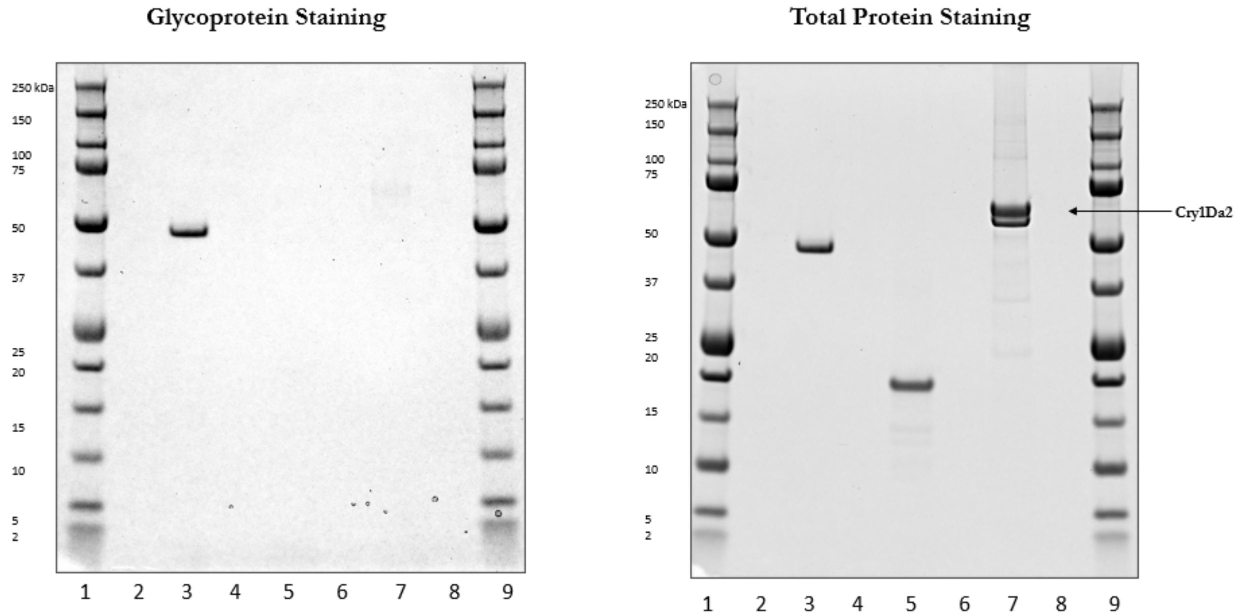
Figure 18. Western Blot Analysis of Cry1Da2 Protein

Protein Glycosylation Analysis

Samples of the DAS1131 maize-derived Cry1Da2 protein and the microbially derived Cry1Da2 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 then 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 determined to be negative for both the DAS1131 maize-derived Cry1Da2 protein and the microbially derived Cry1Da2 protein (Figure 19 and Figure 20, respectively). A very faint band was captured by the imaging system for the maize-derived Cry1Da2 protein sample. This was attributed to non-specific staining which is not due to glycosylation. The horseradish peroxidase positive control was stained 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.

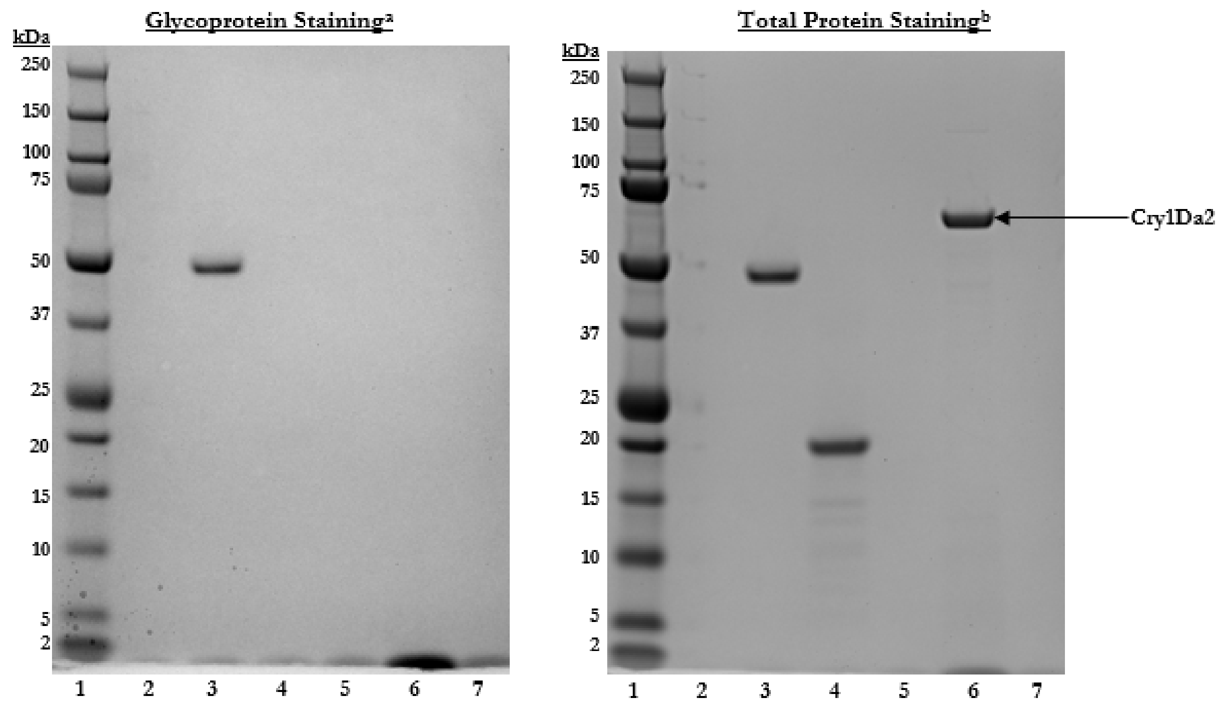


Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	Horseradish Peroxidase (1.0 µg)
4	1X LDS/DTT Sample Buffer Blank
5	Soybean Trypsin Inhibitor (1.0 µg)
6	1X LDS/DTT Sample Buffer Blank
7	DAS1131 Maize-Derived Cry1Da2 Protein
8	1X LDS/DTT Sample 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 19. Glycosylation Analysis of DAS1131 Maize-Derived Cry1Da2 Protein



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[c]
2	1X LDS Sample Buffer Blank
3	Horseradish Peroxidase (1 µg)
4	Soybean Trypsin Inhibitor (1 µg)
5	1X LDS Sample Buffer Blank
6	Microbially Derived Cry1Da2 Protein (1 µg)
7	1X LDS Sample Buffer Blank

Note: kilodalton (kDa) and microgram (µg).

[a] Gel was stained with glycoprotein staining reagent.

[b] Gel was stained with glycoprotein staining reagent followed by staining with Coomassie Blue Reagent for total proteins.

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

Figure 20. Glycosylation Analysis of Microbially Derived Cry1Da2 Protein

Mass Spectrometry Peptide Mapping Analysis

Samples of the DAS1131 maize-derived Cry1Da2 protein and the microbially derived Cry1Da2 protein were subject to SDS-PAGE. Protein bands were stained with Coomassie stain reagent. For the microbially derived Cry1Da2 protein, one sample consisting of bands at the expected molecular weight of the Cry1Da2 protein (approximately 68 kDa) was excised. For the DAS1131 maize-derived Cry1Da2 protein, two samples, one sample consisting of upper band with the molecular weight of approximately 68 kDa, and the other sample consisting of lower band with the molecular weight of approximately 66 kDa, were excised (Figure 17).

The excised Cry1Da2 protein bands were digested with trypsin or chymotrypsin. Digested samples were analyzed with ultra-performance liquid chromatography-mass spectrometry (LC-MS). The resulting MS data were used to search and match the peptides from the expected Cry1Da2 protein sequence, and the combined sequence coverage was calculated.

The identified tryptic and chymotryptic peptides for the DAS1131 maize-derived Cry1Da2 protein are shown in Table 9 - Table 10 and Table 11 - Table 12, respectively. The combined sequence coverage for the upper band (~68 kDa band) of the DAS1131 maize-derived Cry1Da2 protein was 95.9% (578/603) of the expected Cry1Da2 amino acid sequence (Table 13 and Figure 21), and the combined sequence coverage for the lower band (~66 kDa band) of the DAS1131 maize-derived Cry1Da2 protein was 91.0% (549/603) of the expected Cry1Da2 amino acid sequence (Table 13 and Figure 22. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~66 kDa Band)). The N-terminal peptide was identified as MEINNQNQCVPYNCLSNPK from the trypsin digestion of the upper band (Table 9). The results indicated the N-terminal methionine residue of the protein was acetylated. For the lower band, the nineteen amino acids of the N-terminus were not detected by LC-MS (Table 10 and Figure 22. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~66 kDa Band)), indicating N-terminal truncation which was likely due to proteolysis by trypsin-like proteases in planta or during extraction and purification.

The identified tryptic and chymotryptic peptides for the microbially derived Cry1Da2 protein are shown in Table 15 and Table 16, respectively. The combined sequence coverage for the microbially derived Cry1Da2 protein was 93% (560/603) of the expected amino acid sequence (Table 16 and Figure 23). The N-terminal peptide (MEINNQNQCVPY) was identified from the chymotryptic digestion of the microbially derived protein (Table 15). The two amino acid changes (K19Q and R27Q) made to facilitate production were also confirmed by LC-MS analysis of the chymotryptic peptides of the microbially derived protein (Table 15).

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

Table 9. Identified Tryptic Peptides of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~68 kDa Band)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
1–19	2364 044	2364 030	MEINNQNQCVPYNCLSNPK[c]
20–27	957 5089	957 5131	EIILGEER
87–92	763 3862	763 3864	IEEFAR
99–107	1035 559	1035 560	LEGLSNLYK
112–126	1745 843	1745 837	AFSDWEKDPTNPALR
127–148	2624 327	2624 309	EEMRIQFNDMNSALITAIPLFR[d]
131–148	2063 083	2063 087	IQFNDMNSALITAIPLFR
131–172	4757 615	4757 573	IQFNDMNSALITAIPLFRVQNYEVALLSVYVQAANLHLSILR
149–172	2712 501	2712 496	VQNYEVALLSVYVQAANLHLSILR
149–180	3601 956	3601 926	VQNYEVALLSVYVQAANLHLSILRDVSVFGER
173–180	907 4402	907 4400	DVSVFGER
181–191	1309 603	1309 605	WGYDTATINNR
181–215	4159 985	4159 945	WGYDTATINNRYSDLTSLIHVYTNHCVDTYNQGLR
192–215	2868 368	2868 350	YSDLTSLIHVYTNHCVDTYNQGLR
221–230	1311 665	1311 661	FLSDWIVYNR
221–232	1614 832	1614 831	FLSDWIVYNRFR
233–252	2379 310	2379 295	RQLTISVLDIVAFFPNYDIR
234–252	2223 204	2223 194	QLTISVLDIVAFFPNYDIR
253–264	1391 745	1391 741	TYPIQTATQLTR
265–295	3397 728	3397 708	EVYLDLPFINENLSPAASYPTFSAAESAIIR
296–315	2295 160	2295 154	SPHLVDFLNSFTIYTDSLAR
296–328	3845 921	3845 895	SPHLVDFLNSFTIYTDSLARYAYWGGHLVNSFR
316–328	1568 759	1568 752	YAYWGGHLVNSFR
329–337	975 5350	975 5349	TGTTTNLIR
338–343	691 3650	691 3653	SPLYGR
344–363	2170 149	2170 138	EGNTERPVTITASPSVPIFR
364–391	2994 510	2994 493	TLSYITGLDNSNPVAGIEGVEFQNTISR
392–396	665 3887	665 3860	SIYRK
396–422	2841 411	2841 393	KSGPIDSFSELPPQDASVSPAIGYSHR
397–422	2713 316	2713 298	SGPIDSFSELPPQDASVSPAIGYSHR
423–431	1145 567	1145 565	LCHATFLER
437–447	1273 658	1273 657	IAGTVFSWTHR
448–459	1230 584	1230 584	SASPTNEVSPSR
460–467	983 5832	983 5804	ITQIPWVK
460–479	2119 229	2119 215	ITQIPWVKAHTLASGASVIK
468–479	1153 647	1153 646	AHTLASGASVIK
468–491	2325 262	2325 244	AHTLASGASVIKGPGFTGGDILTR
480–491	1189 612	1189 609	GPGFTGGDILTR
480–501	2248 133	2248 127	GPGFTGGDILTRNSMGELGTLR
492–501	1076 530	1076 528	NSMGELGTLR
502–507	679 3660	679 3653	VTFTGR
502–515	1699 909	1699 905	VTFTGRLPQSYYIR
508–515	1038 551	1038 550	LPQSYYIR
516–524	1082 564	1082 562	FRYASVANR
518–524	779 3929	779 3926	YASVANR
530–542	1469 724	1469 719	YSQPPSYGISFPK
543–553	1176 483	1176 545	TMDAGEPLTSR
543–568	2883 439	2883 423	TMDAGEPLTSRSFAHTTLFTPITFSR
554–568	1724 897	1724 889	SFAHTTLFTPITFSR
554–585	3751 889	3751 852	SFAHTTLFTPITFSRAQEEFDLYIQSGVYIDR
569–585	2044 985	2044 974	AQEEFDLYIQSGVYIDR
569–603	4059 026	4059 000	AQEEFDLYIQSGVYIDRIEFIPVTATLEAESDLER
586–603	2032 047	2032 036	IEFIPVTATLEAESDLER

[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 closest to the experimental mass.
[c] The N-terminus was acetylated.
[d] This peptide was modified by methionine oxidation.

Table 10. Identified Tryptic Peptides of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~66 kDa Band)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
20–27	957 5162	957 5131	EIILGEER
87–92	763 3875	763 3864	IEEFAR
99–107	1035 544	1035 560	LEGLSNLYK
112–118	881 3939	881 3919	AFSDWEK
112–126	1745 849	1745 837	AFSDWEKDPTNPALR
119–126	882 4575	882 4559	DPTNPALR
127–148	2640 340	2640 304	EEMRIQFNDMNSALITAIPLFR[c]
131–148	2063 108	2063 087	IQFNDMNSALITAIPLFR
149–172	2712 530	2712 496	VQNYEVALLSVYVQAANLHLSILR
149–180	3601 974	3601 926	VQNYEVALLSVYVQAANLHLSILRDVSVFGER
173–180	907 4424	907 4400	DVSVFGER
181–191	1309 607	1309 605	WGYDTATINNR
192–215	2868 388	2868 350	YSDLTSLIHVYTNHCVDTYNQGLR
221–230	1311 671	1311 661	FLSDWIVYNR
233–252	2379 333	2379 295	RQLTISVLDIVAFFPNYDIR
234–252	2223 225	2223 194	QLTISVLDIVAFFPNYDIR
253–264	1391 750	1391 741	TYPIQTATQLTR
265–295	3397 746	3397 708	EVYLDLPFINENLSPAASYPTFSAAESAIIR
296–315	2295 177	2295 154	SPHLVDFLNSFTIYTDSLAR
296–328	3845 954	3845 895	SPHLVDFLNSFTIYTDSLARYAYWGGHLVNSFR
316–328	1568 762	1568 752	YAYWGGHLVNSFR
329–337	975 5315	975 5349	TGTTTNLIR
338–343	691 3653	691 3653	SPLYGR
344–363	2170 158	2170 138	EGNTERPVTITASPSVPIFR
364–391	2994 531	2994 493	TLSYITGLDNSNPVAGIEGVEFQNTISR
392–396	665 3868	665 3860	SIYRK
396–422	2841 426	2841 393	KSGPIDSFSELPPQDASVSPAIGYSHR
397–422	2713 329	2713 298	SGPIDSFSELPPQDASVSPAIGYSHR
423–431	1145 554	1145 565	LCHATFLER
437–447	1273 662	1273 657	IAGTVFSWTHR
448–459	1230 588	1230 584	SASPTNEVSPSR
460–467	983 5840	983 5804	ITQIPWVK
468–479	1153 648	1153 646	AHTLASGASVIK
468–491	2325 271	2325 244	AHTLASGASVIKGPGFTGGDILTR
480–491	1189 613	1189 609	GPGFTGGDILTR
492–501	1076 533	1076 528	NSMGELGTLR
502–507	679 3652	679 3653	VTFTGR
508–515	1038 551	1038 550	LPQSYYIR
518–524	779 3925	779 3926	YASVANR
530–542	1469 732	1469 719	YSQPPSYGISFPK
543–553	1176 549	1176 545	TMDAGEPLTSR
554–568	1724 901	1724 889	SFAHTTLFTPITFSR
569–585	2044 994	2044 974	AQEEFDLYIQSGVYIDR
586–603	2032 056	2032 036	IEFIPVTATLEAESDLER

[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 closest to the experimental mass.
[c] This peptide was modified by methionine oxidation.

Table 11. Identified Chymotryptic Peptides of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~68 kDa Band)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
1–12	1550 656	1550 649	MEINNQNQCVPY[c]
13–23	1299 689	1299 686	NCLSNPKEIIL
16–23	912 5279	912 5280	SNPKEIIL
16–28	1496 821	1496 820	SNPKEIILGEERL
16–39	2597 379	2597 355	SNPKEIILGEERLETGNTVADISL
24–39	1702 852	1702 837	GEERLETGNTVADISL
24–41	1872 955	1872 943	GEERLETGNTVADISLGL
65–75	1265 616	1265 608	GFIGPSQWDIF
73–82	1188 653	1188 639	DIFLAQIEQL
76–90	1815 986	1815 973	LAQIEQLISQRIEEF
77–90	1702 900	1702 889	AQIEQLISQRIEEF
83–90	1020 525	1020 524	ISQRIEEF
91–99	1027 590	1027 589	ARNQAISRL
91–102	1326 740	1326 737	ARNQAISRLEGL
91–105	1640 907	1640 896	ARNQAISRLEGLSNL
110–116	879 4221	879 4239	VRAFSDW
114–133	2461 183	2461 170	SDWEKDPTNPALREEMRIQF
117–133	2073 039	2073 031	EKDPTNPALREEMRIQF
134–146	1371 714	1371 707	NDMNSALITAIPL
134–147	1518 788	1518 775	NDMNSALITAIPLF
147–156	1237 650	1237 646	FRVQNYEVAL
148–157	1203 663	1203 661	RVQNYEVALL
158–168	1213 648	1213 646	SVYVQAANLHL
161–168	864 4811	864 4817	VQAANLHL
161–171	1177 686	1177 682	VQAANLHLSIL
167–177	1284 723	1284 719	HLSILRDVSVF
169–177	1034 578	1034 576	SILRDVSVF
172–177	721 3758	721 3759	RDVSVF
178–183	766 3407	766 3398	GERWGY
178–192	1814 840	1814 834	GERWGYDTATINNRY
182–192	1286 590	1286 589	GYDTATINNRY
182–195	1601 740	1601 732	GYDTATINNRYSDL
184–192	1066 506	1066 504	DTATINNRY
184–195	1381 649	1381 647	DTATINNRYSDL
184–198	1682 821	1682 811	DTATINNRYSDLTSL
193–202	1146 595	1146 592	SDLTSLIHVY
196–202	831 4485	831 4491	TSLIHVY
215–221	932 5310	932 5304	RRLEGRF
215–222	1045 616	1045 615	RRLEGRFL
226–231	810 4387	810 4388	IVYNRF
236–245	1076 616	1076 612	TISVLDIVAF
246–262	2040 043	2040 032	FPNYDIRTYPIQTATQL
250–262	1518 811	1518 804	DIRTYPIQTATQL
263–272	1251 655	1251 650	TREVYLDLPF
268–277	1186 630	1186 623	LDLPFINENL
269–286	1994 989	1994 963	DLPFINENLSPAASYPTF
273–286	1522 739	1522 730	INENLSPAASYPTF
287–302	1711 900	1711 889	SAAESAIIRSPHLVDF
287–303	1824 984	1824 973	SAAESAIIRSPHLVDFL
307–316	1201 599	1201 598	TIYTDSLARY
310–316	824 4023	824 4028	TDSLARY
310–318	1058 503	1058 503	TDSLARYAY
319–327	1015 487	1015 488	WGGHLVNSF
320–327	829 4077	829 4083	GGHLVNSF
328–335	862 4514	862 4509	RTGTTTNL
328–340	1428 746	1428 805	RTGTTTNLIRSPL
336–341	747 4277	747 4279	IRSPLY

Table 11. Identified Chymotryptic Peptides of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~68 kDa Band) (continued)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
341–362	2390 245	2390 223	YGREGNTERPVTITASPSVPIF
341–365	2760 474	2760 456	YGREGNTERPVTITASPSVPIFRTL
342–362	2227 174	2227 160	GREGNTERPVTITASPSVPIF
342–365	2597 409	2597 393	GREGNTERPVTITASPSVPIFRTL
366–385	2081 015	2080 995	SYITGLDNSNPVAGIEGVEF
368–385	1830 913	1830 900	ITGLDNSNPVAGIEGVEF
372–385	1446 668	1446 663	DNSNPVAGIEGVEF
386–394	1080 555	1080 556	QNTISRSIY
395–403	1005 525	1005 524	RKSGPIDSF
395–419	2617 323	2617 302	RKSGPIDSFSELPPQDASVSPAIGY
404–419	1629 797	1629 789	SELPPQDASVSPAIGY
420–428	1127 531	1127 529	SHRLCHATF
420–429	1240 616	1240 614	SHRLCHATFL
429–442	1514 861	1514 857	LERISGPRIAGTVF
430–442	1401 778	1401 773	ERISGPRIAGTVF
443–465	2636 326	2636 310	SWTHRSASPTNEVSPSRITQIPW
445–465	2363 212	2363 198	THRSASPTNEVSPSRITQIPW
466–471	667 4013	667 4017	VKAHTL
466–483	1738 986	1738 973	VKAHTLASGASVIKGPGF
466–489	2295 278	2295 259	VKAHTLASGASVIKGPGFTGGDIL
472–483	1089 583	1089 582	ASGASVIKGPGF
472–489	1645 879	1645 868	ASGASVIKGPGFTGGDIL
484–497	1462 716	1462 709	TGGDILTRNSMGEL
484–500	1749 868	1749 857	TGGDILTRNSMGELGTL[d]
490–500	1177 573	1177 576	TRNSMGELGTL
490–504	1680 868	1680 862	TRNSMGELGTLRVTF
505–512	920 4695	920 4716	TGRLPQSY
505–513	1083 538	1083 535	TGRLPQSYY
517–528	1327 667	1327 663	RYASVANRSGTF
519–528	1008 497	1008 499	ASVANRSGTF
529–536	996 4674	996 4665	RYSQPPSY
529–550	2440 192	2440 173	RYSQPPSYGISFPKTMDAGEPL
531–550	2121 027	2121 009	SQPPSYGISFPKTMDAGEPL
537–550	1461 724	1461 717	GISFPKTMDAGEPL
556–566	1247 660	1247 655	AHTTLFTPITF
567–576	1256 568	1256 567	SRAQEEFDLY
577–588	1438 754	1438 746	IQSGVYIDRIEF
577–595	2134 192	2134 167	IQSGVYIDRIEFIPVTATL
583–588	791 4170	791 4177	IDRIEF
583–595	1486 849	1486 840	IDRIEFIPVTATL
583–601	2131 126	2131 105	IDRIEFIPVTATLEAESDL
589–603	1642 841	1642 841	IPVTATLEAESDLER
596–603	947 4204	947 4196	EAESDLER

[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 closest to the experimental mass.
[c] The N-terminus was acetylated.
[d] This peptide was modified by methionine oxidation.

Table 12. Identified Chymotryptic Peptides of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~66 kDa Band)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
29–44	1662 871	1662 847	ETGNTVADISLGLINF
76–90	1816 003	1815 973	LAQIEQLISQRIEEF
83–90	1020 528	1020 524	ISQRIEEF
91–99	1027 592	1027 589	ARNQAISRL
91–102	1326 747	1326 737	ARNQAISRLEGL
91–105	1640 908	1640 896	ARNQAISRLEGLSNL
107–113	881 5134	881 5123	KVYVRAF
110–116	879 4247	879 4239	VRAFSDW
114–133	2461 195	2461 170	SDWEKDPTNPALREEMRIQF
117–133	2073 050	2073 031	EKDPTNPALREEMRIQF
134–146	1371 720	1371 707	NDMNSALITAIPL
134–147	1518 794	1518 775	NDMNSALITAIPLF
147–156	1237 654	1237 646	FRVQNYEVAL
148–157	1203 667	1203 661	RVQNYEVALL
158–168	1213 653	1213 646	SVYVQAANLHL
161–171	1177 690	1177 682	VQAANLHLSIL
169–177	1034 582	1034 576	SILRDVSVF
178–183	766 3401	766 3398	GERWGY
178–192	1814 856	1814 834	GERWGYDTATINNRY
182–192	1286 599	1286 589	GYDTATINNRY
182–195	1601 750	1601 732	GYDTATINNRYSDL
184–192	1066 507	1066 504	DTATINNRY
184–198	1682 831	1682 811	DTATINNRYSDLTSL
193–202	1146 601	1146 592	SDLTSLIHVY
203–210	1008 400	1008 397	TNHCVDTY
203–214	1420 613	1420 604	TNHCVDTYNQGL
203–217	1845 902	1845 890	TNHCVDTYNQGLRRL
215–221	932 5321	932 5304	RRLEGRF
246–262	2040 066	2040 032	FPNYDIRTYPIQTATQL
250–262	1518 820	1518 804	DIRTYPIQTATQL
263–272	1251 661	1251 650	TREVYLDLPF
268–277	1186 634	1186 623	LDLPFINENL
273–286	1522 748	1522 730	INENLSPAASYPTF
287–302	1711 912	1711 889	SAAESAIIRSPHLVDF
287–303	1825 000	1824 973	SAAESAIIRSPHLVDFL
307–316	1201 605	1201 598	TIYTDSLARY
310–316	824 4029	824 4028	TDSLARY
310–318	1058 509	1058 503	TDSLARYAY
319–327	1015 491	1015 488	WGGHLVNSF
320–327	829 4090	829 4083	GGHLVNSF
328–335	862 4496	862 4509	RTGTTTNL
328–340	1428 808	1428 805	RTGTTTNLIRSPL
328–341	1591 882	1591 868	RTGTTTNLIRSPLY
336–341	747 4275	747 4279	IRSPLY
341–362	2390 256	2390 223	YGREGNTERPVTITASPSVPIF
342–365	2597 425	2597 393	GREGNTERPVTITASPSVPIFRTL
366–385	2081 030	2080 995	SYITGLDNSNPVAGIEGVEF
372–385	1446 679	1446 663	DNSNPVAGIEGVEF
386–394	1080 470	1080 556	QNTISRSIY
395–403	1005 527	1005 524	RKSGPIDSF
395–419	2617 310	2617 302	RKSGPIDSFSELPPQDASVSPAIGY
404–419	1629 806	1629 789	SELPPQDASVSPAIGY
420–428	1127 532	1127 529	SHRLCHATF
420–429	1240 622	1240 614	SHRLCHATFL
429–442	1514 866	1514 857	LERISGPRIAGTVF
430–442	1401 786	1401 773	ERISGPRIAGTVF
445–465	2363 217	2363 198	THRSASPTNEVSPSRITQIPW

Table 12. Identified Chymotryptic Peptides of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~66 kDa Band) (continued)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
466–471	667 4012	667 4017	VKAHTL
466–489	2295 289	2295 259	VKAHTLASGASVIKGPGFTGGDIL
472–483	1089 587	1089 582	ASGASVIKGPGF
472–489	1645 889	1645 868	ASGASVIKGPGFTGGDIL
484–500	1749 878	1749 857	TGGDILTRNSMGELGTL[c]
490–500	1177 581	1177 576	TRNSMGELGTL
490–504	1680 885	1680 862	TRNSMGELGTLRVTF
505–513	1083 540	1083 535	TGRLPQSYY
517–528	1327 677	1327 663	RYASVANRSGTF
519–528	1008 503	1008 499	ASVANRSGTF
529–536	996 4694	996 4665	RYSQPPSY
529–550	2440 207	2440 173	RYSQPPSYGISFPKTMDAGEPL
531–550	2121 044	2121 009	SQPPSYGISFPKTMDAGEPL
537–550	1461 734	1461 717	GISFPKTMDAGEPL
551–560	1119 572	1119 567	TSRSFAHTTL
551–561	1266 645	1266 636	TSRSFAHTTLF
556–561	688 3523	688 3544	AHTTLF
556–566	1247 665	1247 655	AHTTLFTPITF
567–575	1093 509	1093 504	SRAQEEFDL
567–576	1256 575	1256 567	SRAQEEFDLY
577–588	1438 762	1438 746	IQSGVYIDRIEF
577–595	2134 206	2134 167	IQSGVYIDRIEFIPVTATL
583–595	1486 858	1486 840	IDRIEFIPVTATL

[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 closest to the experimental mass.
[c] This peptide was modified by methionine oxidation.

Table 13. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis

Cry1Da2 Protein Band	Molecular Weight (kDa)	Protease	% Coverage	Combined % Sequence Coverage
Upper	~68	Trypsin	86	95.9
		Chymotrypsin	90	
Lower	~66	Trypsin	82	91.0
		Chymotrypsin	83	

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  1 MEINNQNQCV PYNCLSNPKE IILGEERLET GNTVADISLG LINFLYSNFV
 51 PGGGFIVGLL ELIWGFIGPS QWDIFLAQIE QLISQRIEEF ARNQAISRLE
101 GLSNLYKVYV RAFSDWEKDP TNPALREEMR IQFNDMNSAL ITAIPLFRVQ
151 NYEVALLSVY VQAANLHLSI LRDVSVFGER WGYDTATINN RYSDLTSLIH
201 VYTNHCVDTY NQGLRRLEGR FLSDWIVYNR FRRQLTISVL DIVAFFPNYD
251 IRTYPIQTAT QLTREVYLDL PFINENLSPA ASYPTFSAAE SAIIRSPHLV
301 DFLNSFTIYT DSLARYAYWG GHLVNSFRTG TTTNLIRSPL YGREGNTERP
351 VTITASPSVP IFRTLSYITG LDNSNPVAGI EGVEFQNTIS RSIYRKSGPI
401 DSFSELPPQD ASVSPAIGYS HRLCHATFLE RISGPRIAGT VFSWTHRSAS
451 PTNEVSPSRI TQIPWVKAHT LASGASVIKG PGFTGGDILT RNSMGELGTL
501 RVTFTGRLPQ SYYIRFRYAS VANRSGTFRY SQPPSYGISF PKTMDAGEPL
551 TSRSFAHTTL FTPITFSRAQ EEFDLYIQSG VYIDRIEFIP VTATLEAESD
601 LER
```

Red type	Bold red type indicates maize-derived Cry1Da2 peptides identified using LC-MS analysis. The identified sequence is identical to the Cry1Da2 sequence deduced by the translation of the *cry*1Da2 gene and the expected sequence used in LC-MS analysis.
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 21. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~68 kDa Band)

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  1 MEINNQNQCV PYNCLSNPKE IILGEERLET GNTVADISLG LINFLYSNFV
 51 PGGGFIVGLL ELIWGFIGPS QWDIFLAQIE QLISQRIEEF ARNQAISRLE
101 GLSNLYKVYV RAFSDWEKDP TNPALREEMR IQFNDMNSAL ITAIPLFRVQ
151 NYEVALLSVY VQAANLHLSI LRDVSVFGER WGYDTATINN RYSDLTSLIH
201 VYTNHCVDTY NQGLRRLEGR FLSDWIVYNR FRRQLTISVL DIVAFFPNYD
251 IRTYPIQTAT QLTREVYLDL PFINENLSPA ASYPTFSAAE SAIIRSPHLV
301 DFLNSFTIYT DSLARYAYWG GHLVNSFRTG TTTNLIRSPL YGREGNTERP
351 VTITASPSVP IFRTLSYITG LDNSNPVAGI EGVEFQNTIS RSIYRKSGPI
401 DSFSELPPQD ASVSPAIGYS HRLCHATFLE RISGPRIAGT VFSWTHRSAS
451 PTNEVSPSRI TQIPWVKAHT LASGASVIKG PGFTGGDILT RNSMGELGTL
501 RVTFTGRLPQ SYYIRFRYAS VANRSGTFRY SQPPSYGISF PKTMDAGEPL
551 TSRSFAHTTL FTPITFSRAQ EEFDLYIQSG VYIDRIEFIP VTATLEAESD
601 LER
```


Red type	Bold red type indicates maize-derived Cry1Da2 peptides identified using LC-MS analysis. The identified sequence is identical to the Cry1Da2 sequence deduced by the translation of the *cry*1Da2 gene and the expected sequence used in LC-MS analysis.
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 22. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~66 kDa Band)

Table 14. Identified Tryptic Peptides of Microbially Derived Cry1Da2 Protein Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
87–92	763 3888	763 3864	IEEFAR
93–98	687 3682	687 3664	NQAISR
99–107	1035 5595	1035 5600	LEGLSNLYK
112–118	881 3952	881 3919	AFSDWEK
112–126	1745 8443	1745 8373	AFSDWEKDPTNPALR
119–130	1427 6882	1427 6827	DPTNPALREEMR
131–148	2079 0868	2079 0823	IQFNDMNSALITAIPLFR (Oxidation-M)[c]
173–180	907 4429	907 4400	DVSVFGER
181–191	1309 6083	1309 6051	WGYDTATINNR
192–215	2868 3605	2868 3501	YSDLTSLIHVYTNHCVDTYNQGLR
221–230	1311 6620	1311 6612	FLSDWIVYNR
253–264	1391 7433	1391 7409	TYPIQTATQLTR
296–315	2295 1602	2295 1535	SPHLVDFLNSFTIYTDSLAR
316–328	1568 7569	1568 7524	YAYWGGHLVNSFR
329–337	975 5376	975 5349	TGTTTNLIR
338–343	691 3682	691 3653	SPLYGR
344–363	2170 1487	2170 1382	EGNTERPVTITASPSVPIFR
364–391	2994 495	2994 4934	TLSYITGLDNSNPVAGIEGVEFQNTISR
392–396	665 3882	665 3860	SIYRK
396–422	2841 4058	2841 3933	KSGPIDSFSELPPQDASVSPAIGYSHR
397–422	2713 3125	2713 2984	SGPIDSFSELPPQDASVSPAIGYSHR
423–431	1145 5701	1145 5652	LCHATFLER
437–447	1273 6632	1273 6568	IAGTVFSWTHR
448–459	1230 5866	1230 584	SASPTNEVSPSR
460–467	983 5831	983 5804	ITQIPWVK
468–479	1153 6498	1153 6455	AHTLASGASVIK
468–491	2325 2535	2325 2441	HTLASGASVIKGPGFTGGDILTR
480–491	1189 6126	1189 6092	GPGFTGGDILTR
480–501	2248 1367	2248 1270	GPGFTGGDILTRNSMGELGTLR
492–501	1076 5306	1076 5284	NSMGELGTLR
502–507	679 3678	679 3653	VTFTGR
508–515	1038 5525	1038 5498	LPQSYYIR
518–524	779 3948	779 3926	YASVANR
530–542	1469 7224	1469 7191	YSQPPSYGISFPK
543–553	1176 5472	1176 5445	TMDAGEPLTSR
554–568	1724 8898	1724 8886	SFAHTTLFTPITFSR
569–585	2044 9786	2044 9742	AQEEFDLYIQSGVYIDR
586–603	2032 0397	2032 0364	IEFIPVTATLEAESDLER

[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 closest to the experimental mass.
[c] This peptide was modified by methionine oxidation (Oxidation-M).

Table 15. Identified Chymotryptic Peptides of Microbially Derived Cry1Da2 Protein Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
1–12	1508 6429	1508 6388	MEINNQNQCVPY
2–12	1377 6024	1377 5983	EINNQNQCVPY
16–39	2569 2954	2569 2759	SNPQEIILGEEQLETGNTVADISL
65–72	890 4297	890 4287	GFIGPSQW
65–75	1265 6107	1265 6081	GFIGPSQWDIF
73–82	1188 6431	1188 6390	DIFLAQIEQL
76–90	1815 9827	1815 9730	LAQIEQLISQRIEEF
83–90	1020 5245	1020 5240	ISQRIEEF
91–99	1027 5899	1027 5886	ARNQAISRL
114–133	2461 1853	2461 1696	SDWEKDPTNPALREEMRIQF
117–133	2089 0344	2089 0262	EKDPTNPALREEMRIQF (Oxidation-M)[c]
134–146	1371 7119	1371 7068	NDMNSALITAIPL
134–147	1534 7748	1534 7701	NDMNSALITAIPLF (Oxidation-M)[c]
147–152	825 4143	825 4133	FRVQNY
147–156	1237 6472	1237 6455	FRVQNYEVAL
148–157	1203 6670	1203 6611	RVQNYEVALL
158–166	963 5031	963 5025	SVYVQAANL
158–168	1213 6503	1213 6455	SVYVQAANLHL
161–168	864 4834	864 4817	VQAANLHL
161–171	1177 6862	1177 6819	VQAANLHLSIL
167–177	1284 7254	1284 7190	HLSILRDVSVF
169–177	1034 5787	1034 5760	SILRDVSVF
172–177	721 3771	721 3759	RDVSVF
178–183	766 3402	766 3398	GERWGY
178–192	1814 8405	1814 8336	GERWGYDTATINNRY
182–192	1286 5918	1286 5891	GYDTATINNRY
182–195	1601 7410	1601 7321	GYDTATINNRYSDL
184–192	1066 5057	1066 5043	DTATINNRY
184–195	1381 6530	1381 6474	DTATINNRYSDL
184–198	1682 8221	1682 8111	DTATINNRYSDLTSL
193–202	1146 5961	1146 5921	SDLTSLIHVY
196–202	831 4513	831 4491	TSLIHVY
203–210	1008 3972	1008 3971	TNHCVDTY
215–222	1045 6170	1045 6145	RRLEGRFL
218–225	1008 4686	1008 4665	EGRFLSDW
241–249	1084 5263	1084 5229	DIVAFFPNY
246–262	2040 0451	2040 0316	FPNYDIRTYPIQTATQL
250–262	1518 8127	1518 8042	DIRTYPIQTATQL
263–272	1251 6549	1251 6499	TREVYLDLPF
268–277	1186 6271	1186 6234	LDLPFINENL
273–286	1522 7415	1522 7303	INENLSPAASYPTF
278–286	939 4362	939 4338	SPAASYPTF
287–302	1711 8989	1711 8893	SAAESAIIRSPHLVDF
287–303	1824 9831	1824 9734	SAAESAIIRSPHLVDFL
307–316	1201 6011	1201 5979	TIYTDSLARY
310–316	824 4030	824 4028	TDSLARY
317–323	802 3767	802 3762	AYWGGHL
319–327	1015 4905	1015 4876	WGGHLVNSF
320–327	829 4095	829 4083	GGHLVNSF
328–335	862 4530	862 4509	RTGTTTNL
328–340	1428 8082	1428 8049	RTGTTTNLIRSPL
336–341	747 4292	747 4279	IRSPLY
341–362	2390 2430	2390 2230	YGREGNTERPVTITASPSVPIF
341–365	2760 4754	2760 4559	YGREGNTERPVTITASPSVPIFRTL
342–362	2227 1778	2227 1597	GREGNTERPVTITASPSVPIF
342–365	2597 4093	2597 3925	GREGNTERPVTITASPSVPIFRTL
366–385	2081 0109	2080 9953	SYITGLDNSNPVAGIEGVEF
368–385	1830 9116	1830 9000	ITGLDNSNPVAGIEGVEF

Table 15. Identified Chymotryptic Peptides of Microbially Derived Cry1Da2 Protein Using LC-MS Analysis (continued)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
372–385	1446 6722	1446 6627	DNSNPVAGIEGVEF
386–394	1080 5583	1080 5564	QNTISRSIY
395–403	1005 5258	1005 5243	RKSGPIDSF
395–419	2617 3258	2617 3024	RKSGPIDSFSELPPQDASVSPAIGY
404–419	1629 7979	1629 7886	SELPPQDASVSPAIGY
420–428	1127 5321	1127 5294	SHRLCHATF
429–442	1514 8620	1514 8569	LERISGPRIAGTVF
430–442	1401 7771	1401 7728	ERISGPRIAGTVF
445–465	2363 2088	2363 1982	THRSASPTNEVSPSRITQIPW
472–483	1089 5840	1089 5819	ASGASVIKGPGF
472–489	1645 8806	1645 8676	ASGASVIKGPGFTGGDIL
484–500	1733 8741	1733 8618	TGGDILTRNSMGELGTL
490–500	1177 5784	1177 5761	TRNSMGELGTL
490–504	1680 8698	1680 8617	TRNSMGELGTLRVTF
498–504	792 4507	792 4494	GTLRVTF
505–512	920 4726	920 4716	TGRLPQSY
505–513	1083 5370	1083 5349	TGRLPQSYY
517–528	1327 6756	1327 6633	RYASVANRSGTF
519–528	1008 5007	1008 4989	ASVANRSGTF
529–536	996 4686	996 4665	RYSQPPSY
529–550	2440 1946	2440 1733	RYSQPPSYGISFPKTMDAGEPL
537–550	1461 7265	1461 7174	GISFPKTMDAGEPL
556–561	688 3543	688 3544	AHTTLF
556–566	1247 6604	1247 6550	AHTTLFTPITF
567–573	865 3932	865 3930	SRAQEEF
567–575	1093 5068	1093 5040	SRAQEEFDL
567–576	1256 5708	1256 5673	SRAQEEFDLY
583–588	791 4180	791 4177	IDRIEF
583–595	1486 8474	1486 8395	IDRIEFIPVTATL
589–603	1642 8505	1642 8414	IPVTATLEAESDLER
596–603	947 4215	947 4196	EAESDLER

[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 closest to the experimental mass.
[c] This peptide was modified by methionine oxidation (Oxidation-M).

Table 16. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of Microbially Derived Cry1Da2 Protein Using LC-MS Analysis

Protease	% Coverage	Combined % Coverage
Trypsin	69	93
Chymotrypsin	85	

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  1 MEINNQNQCV PYNCLSNPQE IILGEEQLET GNTVADISLG LINFLYSNFV
 51 PGGGFIVGLL ELIWGFIGPS QWDIFLAQIE QLISQRIEEF ARNQAISRLE
101 GLSNLYKVYV RAFSDWEKDP TNPALREEMR IQFNDMNSAL ITAIPLFRVQ
151 NYEVALLSVY VQAANLHLSI LRDVSVFGER WGYDTATINN RYSDLTSLIH
201 VYTNHCVDTY NQGLRRLEGR FLSDWIVYNR FRRQLTISVL DIVAFFPNYD
251 IRTYPIQTAT QLTREVYLDL PFINENLSPA ASYPTFSAAE SAIIRSPHLV
301 DFLNSFTIYT DSLARYAYWG GHLVNSFRTG TTTNLIRSPL YGREGNTERP
351 VTITASPSVP IFRTLSYITG LDNSNPVAGI EGVEFQNTIS RSIYRKSGPI
401 DSFSELPPQD ASVSPAIGYS HRLCHATFLE RISGPRIAGT VFSWTHRSAS
451 PTNEVSPSRI TQIPWVKAHT LASGASVIKG PGFTGGDILT RNSMGELGTL
501 RVTFTGRLPQ SYYIRFRYAS VANRSGTFRY SQPPSYGISF PKTMDAGEPL
551 TSRSFAHTTL FTPITFSRAQ EEFDLYIQSG VYIDRIEFIP VTATLEAESD
601 LER
```

Red type	Red type indicates microbially-derived Cry1Da2 peptides identified using LC-MS analysis.
Amino acid residue abbreviations	alanine (A), cysteine (C), 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 23. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of Microbially Derived Cry1Da2 Protein by LC-MS Analysis

N-Terminal Amino Acid Sequence Analysis

As stated previously in the Mass Spectrometry Peptide Mapping Analysis section, the N-terminal peptide (MEINNQNQCVPYNCLSNPK) of the DAS1131 maize-derived Cry1Da2 was identified with the LC-MS from the trypsin digestion of the upper band (Table 9 and Table 17; Figure 21. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of DAS1131 Maize-Derived Cry1Da2 Protein Using LC-MS Analysis (~68 kDa Band)). For the lower band of the DAS1131 maize-derived Cry1Da2, the nineteen amino acids of the N-terminus were not detected by LC-MS, indicating N-terminal truncation which was likely due to proteolysis by trypsin-like proteases in planta or during extraction and purification (Table 10 and Figure 22). The N-terminal peptide (MEINNQNQCVPY) was identified from the chymotryptic digestion of the microbially derived protein using LC-MS analysis (Table 15 and Figure 23). In addition, the analysis of the microbially derived Cry1Da2 protein using Edman sequencing identified an N-terminal sequence (MEINNQNQ_V), matching amino acid residues 1-8 and 10 of the expected sequence (Table 17).

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

Table 17. N-Terminal Amino Acid Sequence of Cry1Da2 Protein

Description		Amino Acid Sequence
DAS1131 maize-derived Cry1Da2 protein	Deduced Sequence[a]	M-E-I-N-N-Q-N-Q-C-V-P-Y-N-C-L-S-N-P-K
	Identified Sequence[b]	M-E-I-N-N-Q-N-Q-C-V-P-Y-N-C-L-S-N-P-K
Microbially derived Cry1Da2 protein	Deduced Sequence	M-E-I-N-N-Q-N-Q-C-V-P-Y
	Identified Sequence	M-E-I-N-N-Q-N-Q-C-V-P-Y

[a] The deduced Cry1Da2 protein sequence is a result of the translation of the gene.

[b] The identified N-terminal sequence from the upper band (~68 kDa) of the DAS1131 maize-derived Cry1Da2 protein

Bioactivity Assay

The bioactivity of the microbially derived Cry1Da2 protein was evaluated by conducting a 7-day bioassay using *Spodoptera frugiperda* (fall armyworm; Lepidoptera: Noctuidae), a species sensitive to the Cry1Da2 protein.

Bioactivity analysis demonstrated that the microbially derived Cry1Da2 protein had insecticidal activity toward the target insect, *S. frugiperda* (Table 18). The biological activity of the test diet containing the 50 ng Cry1Da2 protein was demonstrated by the increased mortality and decreased weight of *S. frugiperda* fed the test diet.

Table 18. Microbially Derived Cry1Da2 Protein Bioactivity Assay Using *Spodoptera frugiperda*

Treatment	Treatment Description	Protein Concentration (ng Cry1Da2/mg)	Total Number of Observations	Mortality (%)	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
						Mean ± Standard Deviation	Range
A	Bioassay Control Diet	0	20	0	20	37.3 ± 8.5	22.0-50.4
B	Test Diet	50	20	95	1	10.9[a]	NA

Note: The concentration of the Cry1Da2 protein in Treatment B was based on the wet weight of the artificial diet. The summary of *Spodoptera frugiperda* mortality data consisted of the calculation of dead larvae divided by the total number of observed larvae at the end of the study and multiplied by 100. Not applicable (NA); there was only one surviving organism in the test diet group.

[a] The reported mean is the weight value of the one surviving organism after the 7-day feeding period.

Allergenicity and Toxicity Analyses of the Cry1Da2 Protein

A weight-of-evidence approach was applied to determine the allergenic and toxic potential of the Cry1Da2 protein expressed in DAS1131 maize, including an assessment of the following: a bioinformatic comparison of the amino acid sequence of Cry1Da2 protein to known or putative protein allergen and toxin sequences, evaluation of the stability of the Cry1Da2 protein using *in vitro* gastric and intestinal digestion models, determination of the Cry1Da2 protein glycosylation status, evaluation of the heat lability of the Cry1Da2 protein using a sensitive insect bioassay, and an evaluation of acute toxicity in mice following oral exposure to Cry1Da2 protein (Carlson *et al.*, 2019).

Bioinformatic Analysis of Homology to Known or Putative Allergens (PHI-2021-073/201 study)

Assessing newly expressed proteins for potential cross-reactivity with known or putative allergens is an important part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically-modified plant products (Codex Alimentarius Commission, 2003). A bioinformatic assessment of the Cry1Da2 protein sequence for potential cross-reactivity with known or putative allergens was conducted according to relevant guidelines (Codex Alimentarius Commission, 2003; FAO/WHO, 2001).

Two separate searches for the Cry1Da2 protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2021 database (January 2021) available at http://comparedatabase.org. This peer-reviewed database is a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins, and Bioinformatics (PATB) Committee and is comprised of 2,348 sequences. The first search used the Cry1Da2 protein sequence as the query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the allergen sequences. The search was conducted using default parameters, except the *E*-score threshold was set to 10^{-4}. An *E*-score threshold of 10^{-4} has been shown to be an appropriate value for allergenicity searches (Mirsky *et al.*, 2013). The generated alignments were examined to identify any that are 80 residues or longer and possess a sequence identity of greater than 35%. The second search used the FUZZPRO program (Emboss Package v6.4.0) to identify any contiguous 8-residue identical matches between the Cry1Da2 protein sequence and the allergen sequences.

Results of the search of the Cry1Da2 protein sequence against the COMPARE database of known and putative allergens found no alignments that were 80 residues or longer with a sequence identity of greater than 35%. No contiguous 8-residue matches between the Cry1Da2 protein sequence and the allergen sequences were identified in the second search.

Collectively, these data indicate that no allergenicity concern arose from the bioinformatics assessment of the Cry1Da2 protein.

Bioinformatic Analysis of Homology to Known or Putative Toxins (PHI-2021-208/211 study)

Assessing newly expressed proteins for potential toxicity is a critical part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically modified plant products (Codex Alimentarius Commission, 2003). The potential toxicity of the Cry1Da2 protein was assessed by comparison of its sequence to the sequences in an internal toxin database. The

internal toxin database is a subset of sequences found in UniProtKB/Swiss-Prot (https://www.uniprot.org/). To produce the internal toxin database, the manually annotated proteins in UniProtKB/Swiss-Prot are filtered for molecular function by keywords that could imply toxicity or adverse health effects (e.g., toxin, hemagglutinin, vasoactive, etc.). The internal toxin database is updated annually. The search between the Cry1Da2 protein sequence and protein sequences in the internal toxin database was conducted with BLASTP using default parameters, except that low complexity filtering was turned off, the *E*-value threshold was set to 10^{-4}, and unlimited alignments were returned.

No alignments with an *E*-value ≤ 10^{-4} were returned between the Cry1Da2 protein sequence and any protein sequence in the internal toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the Cry1Da2 protein.

Thermolability Analysis (PHI-2021-072 study)

Thermal stability of the Cry1Da2 protein was characterized by determining the biological activity of the heat-treated Cry1Da2 protein when incorporated in an artificial diet and fed to *Spodoptera frugiperda*, an insect sensitive to the test substance. Purified Cry1Da2 protein was incubated at various temperatures for 30-35 minutes before incorporation into the artificial diet. Larvae were exposed via oral ingestion to the diets in a 7-day bioassay. A positive control diet containing the unheated Cry1Da2 protein and a bioassay control diet containing ultrapure water were included in the bioassay to verify assay performance. After seven days, statistical analyses were conducted to evaluate *S. frugiperda* mortality of the heat-treated test groups relative to the unheated test group.

The results demonstrated that Cry1Da2 protein heat-treated at a temperature of 75 °C or higher was inactive against *S. frugiperda* when incorporated in an artificial insect diet (Table 19).

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

Table 19. Biological Activity of Heat-Treated Cry1Da2 Protein in Artificial Diet Fed to *Spodoptera frugiperda*

Treatment	Treatment Description	Test Dosing Solution Incubation Condition	Total Number of Observations	Mortality (%)	Fisher's Test P-Value	Number of Surviving Organisms	Weight of Surviving Organisms (mg)	
							Mean ± Standard Deviation	Range
1	Bioassay Control Diet	NA	20	0	--	20	41.1 ± 9.83	15.3 - 53.2
2	Unheated Control Diet	Unheated	20	85.0	--	3	24.4 ± 19.0	3.0 - 39.3
3	Test Diet	25 °C	20	100	1.0000	0	NA	NA
4	Test Diet	50 °C	20	90.0	0.8292	2	5.25 ± 7.42	0.0 - 10.5
5	Test Diet	75 °C	20	0	<0.0001[a]	20	44.2 ± 7.83	25.6 - 55.9
6	Test Diet	95 °C	20	0	<0.0001[a]	20	42.4 ± 9.45	7.9 - 52.0

Note: The unheated control diet and the test diets contained a targeted concentration of 16 ng Cry1Da2 protein per mg diet wet weight. Not applicable (NA).

[a] A statistically significant difference (P-value < 0.05) was observed in comparison to Treatment 2.

Digestibility Analysis with Simulated Gastric Fluid (PHI-2021-098 study)

Simulated gastric fluid (SGF) containing pepsin at pH ~1.2 was used to assess the susceptibility of the Cry1Da2 protein to proteolytic digestion by pepsin *in vitro*. The Cry1Da2 protein was incubated in SGF for 0, 0.5, 1, 2, 5, 10, 20, and 60 minutes. A positive control (bovine serum albumin) and a negative control (β-lactoglobulin) were included in the assay and were incubated in SGF for 0, 1, and 60 minutes. After incubation in SGF, the samples were analyzed by SDS-PAGE. Coomassie-based stain or western blot was used to detect protein bands.

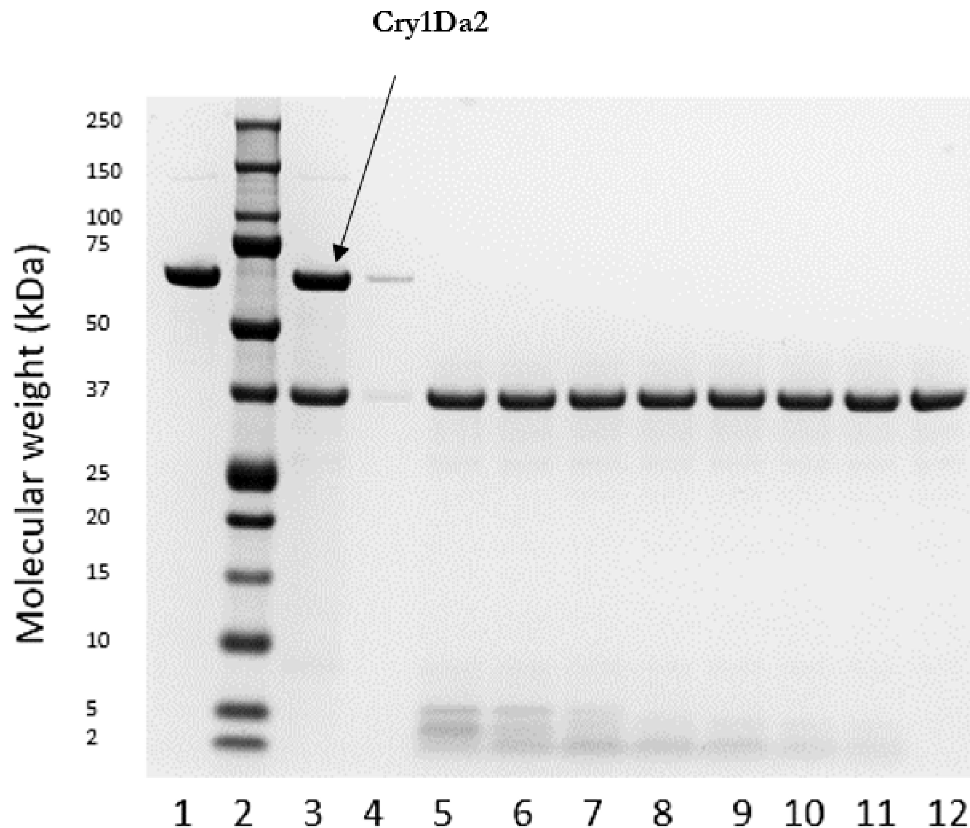
A summary of the SGF results is provided in Table 20. The Cry1Da2 protein migrating at ~68 kDa was digested (within 0.5 minutes) in SGF as demonstrated by both SDS-PAGE and western blot analysis (Figure 24 and Figure 25). A weak band migrating at slightly less than the full length Cry1Da2 protein visible on the western blot after 0.5 minutes digestion was digested within 1 minute in SGF. A band migrating at ~15 kDa visible on the western blot was digested within 5 minutes in SGF. On the SDS-PAGE gel, low molecular weight weak bands (~2-5 kDa) remained detectable in the Cry1Da2 protein samples for up to 60 minutes in SGF. As expected, the BSA control was digested within 1 minute and low molecular weight bands remained detectable at 60 minutes. The β-lactoglobulin control remained detectable after 60 minutes in SGF.

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

Table 20. Summary of Cry1Da2 Protein In Vitro Pepsin Resistance Analysis

Protein	Approximate Molecular Weight (kDa)	Digestion Time Determined by SDS-PAGE (minutes)	Digestion Time Determined by Western Blot (minutes)
Cry1Da2	68	< 0.5	< 0.5
Bovine Serum Albumin	66	< 1	NA
β-lactoglobulin	18	> 60	NA

Note: kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and not applicable (NA).

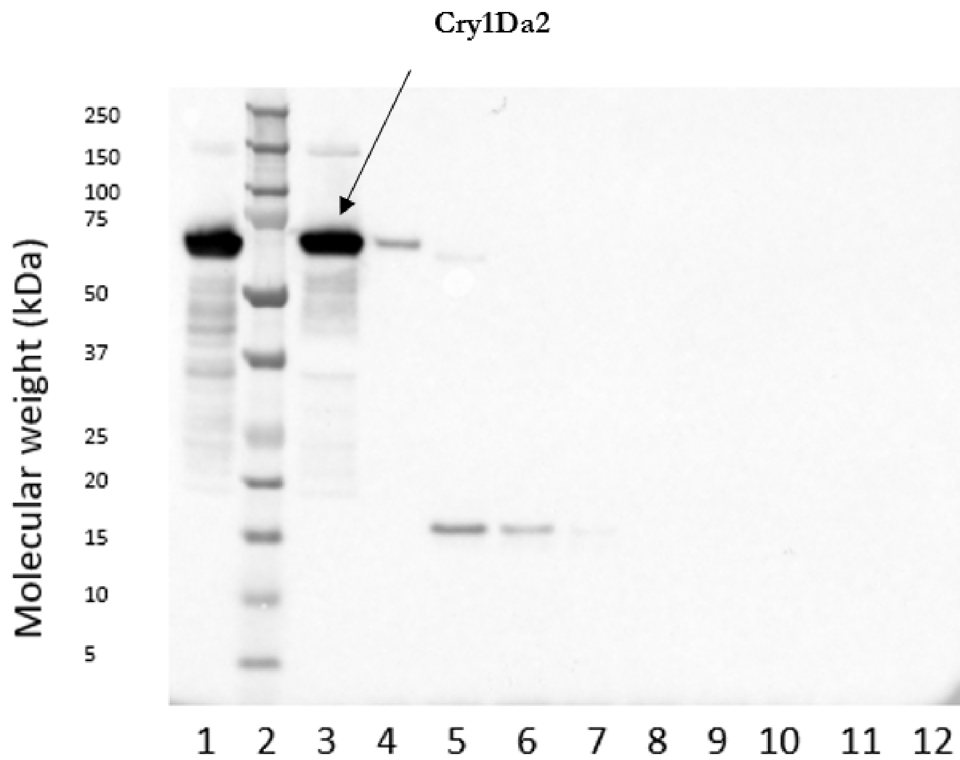


Lane	Sample Descriptions
1	Cry1Da2 protein in water (no SGF), Time 0
2	Pre-stained protein molecular weight marker[a]
3	Cry1Da2 protein in SGF, Time 0
4	Cry1Da2 protein in SGF, Time 0; 1:20 dilution
5	Cry1Da2 protein in SGF, 0.5 minutes
6	Cry1Da2 protein in SGF, 1 minute
7	Cry1Da2 protein in SGF, 2 minutes
8	Cry1Da2 protein in SGF, 5 minutes
9	Cry1Da2 protein in SGF, 10 minutes
10	Cry1Da2 protein in SGF, 20 minutes
11	Cry1Da2 protein in SGF, 60 minutes
12	SGF Control, 60 minutes

Note: kilodalton (kDa), simulated gastric fluid (SGF), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

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

Figure 24. SDS-PAGE Analysis of Cry1Da2 Protein in Simulated Gastric Fluid Digestion Time Course



Lane	Sample Descriptions
1	Cry1Da2 protein in water (no SGF), Time 0
2	Pre-stained protein molecular weight marker[a]
3	Cry1Da2 protein in SGF, Time 0
4	Cry1Da2 protein in SGF, Time 0; 1:100 dilution
5	Cry1Da2 protein in SGF, 0.5 minutes
6	Cry1Da2 protein in SGF, 1 minute
7	Cry1Da2 protein in SGF, 2 minutes
8	Cry1Da2 protein in SGF, 5 minutes
9	Cry1Da2 protein in SGF, 10 minutes
10	Cry1Da2 protein in SGF, 20 minutes
11	Cry1Da2 protein in SGF, 60 minutes
12	SGF Control, 60 minutes

Note: kilodalton (kDa) and simulated gastric fluid (SGF).

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

Figure 25. Western Blot Analysis of Cry1Da2 Protein in Simulated Gastric Fluid Digestion Time Course

Digestibility Analysis with Simulated Intestinal Fluid (PHI-2021-101 study)

Simulated intestinal fluid (SIF) containing pancreatin at ~pH 7.5 was used to assess the susceptibility of the Cry1Da2 protein to proteolytic digestion by pancreatin *in vitro*. The Cry1Da2 protein was incubated in SIF for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 minutes. A positive control (β-lactoglobulin) and a negative control (bovine serum albumin) were included in the assay and were incubated in SIF for 0, 1, and 60 minutes. After incubation in SIF, the samples were analyzed by SDS-PAGE. Coomassie-based stain or western blot was used to detect protein bands.

A summary of the SIF assay results is provided in Table 21. The Cry1Da2 protein migrating at ~68 kDa was digested into fragments migrating from slightly smaller than the full-length protein to ~20 kDa within 0.5 minutes in SIF as was evident on both the stained SDS-PAGE gel and western blot (Figure 26 and Figure 27, respectively). These smaller fragments remained detectable for up to 60 minutes in SIF. As expected, the β-lactoglobulin control was digested within 60 minutes in SIF. The BSA control remained detectable after 60 minutes in SIF.

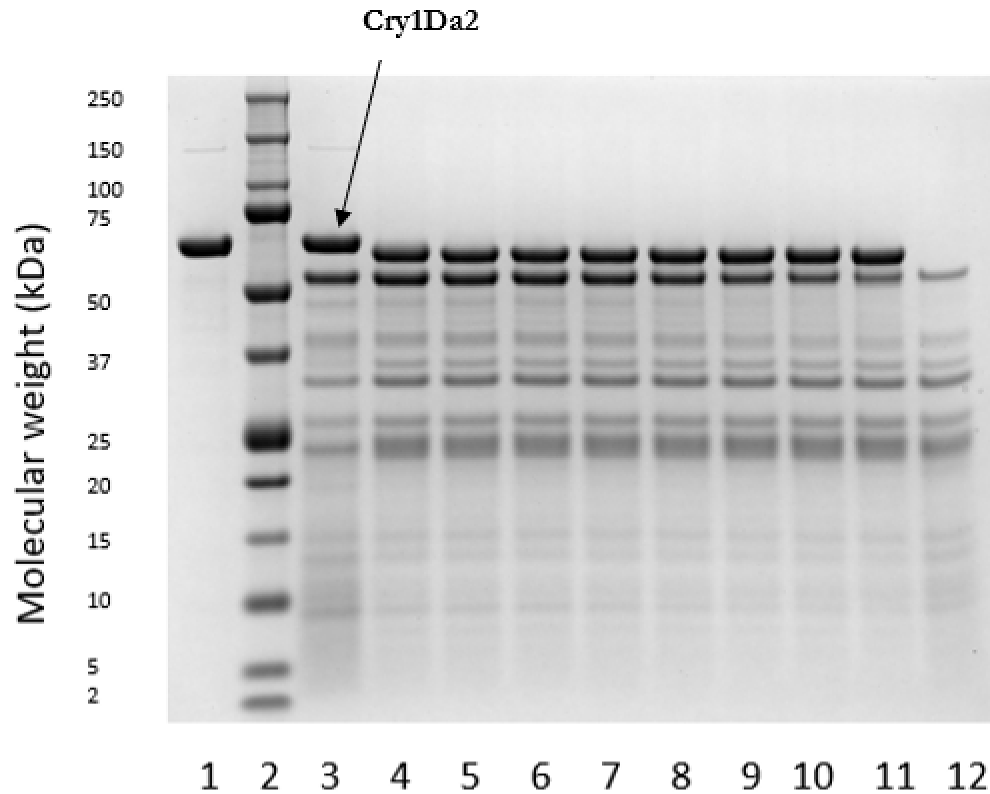
Additional details regarding SIF analytical methods are provided in Appendix E.

Table 21. Summary of Cry1Da2 Protein *In Vitro* Pancreatin Resistance Analyses

Protein	Approximate Molecular Weight (kDa)	Digestion Time Determined by SDS-PAGE (minutes)	Digestion Time Determined by Western Blot (minutes)
Cry1Da2	68	< 0.5[a]	< 0.5
β-lactoglobulin	18	< 60	NA
Bovine Serum Albumin	66	> 60	NA

Note: kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and not applicable (NA).

[a] The full-length Cry1Da2 protein migrating at ~68 kDa was digested into fragments migrating from slightly smaller than the full-length protein to ~20 kDa; these smaller fragments remained detectable for up to 60 minutes in simulated intestinal fluid (SIF).

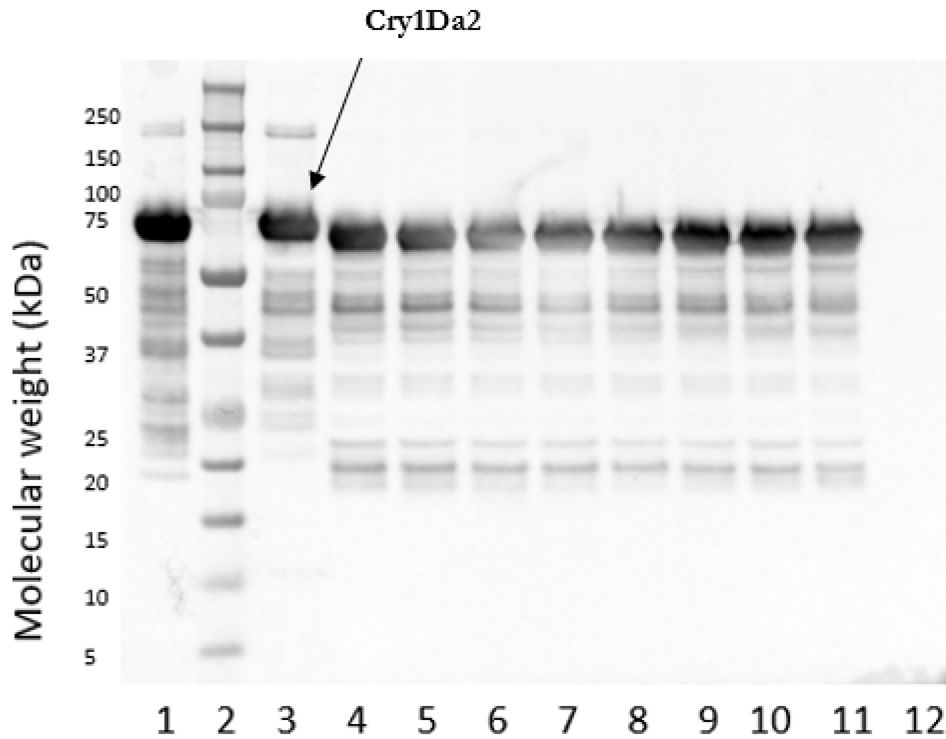


Lane	Sample Descriptions
1	Cry1Da2 protein in water (no SIF), Time 0
2	Pre-stained protein molecular weight marker[a]
3	Cry1Da2 protein in SIF, Time 0
4	Cry1Da2 protein in SIF, 0.5 minutes
5	Cry1Da2 protein in SIF, 1 minute
6	Cry1Da2 protein in SIF, 2 minutes
7	Cry1Da2 protein in SIF, 5 minutes
8	Cry1Da2 protein in SIF, 10 minutes
9	Cry1Da2 protein in SIF, 20 minutes
10	Cry1Da2 protein in SIF, 30 minutes
11	Cry1Da2 protein in SIF, 60 minutes
12	SIF Control, 60 minutes

Note: kilodalton (kDa), simulated intestinal fluid (SIF), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

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

Figure 26. SDS-PAGE Analysis of Cry1Da2 Protein in Simulated Intestinal Fluid Digestion Time Course



Lane	Sample Descriptions
1	Cry1Da2 protein in water (no SIF), Time 0
2	Pre-stained protein molecular weight marker[a]
3	Cry1Da2 protein in SIF, Time 0
4	Cry1Da2 protein in SIF, 0.5 minutes
5	Cry1Da2 protein in SIF, 1 minute
6	Cry1Da2 protein in SIF, 2 minutes
7	Cry1Da2 protein in SIF, 5 minutes
8	Cry1Da2 protein in SIF, 10 minutes
9	Cry1Da2 protein in SIF, 20 minutes
10	Cry1Da2 protein in SIF, 30 minutes
11	Cry1Da2 protein in SIF, 60 minutes
12	SIF Control, 60 minutes

Note: kilodalton (kDa) and simulated intestinal fluid (SIF).

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

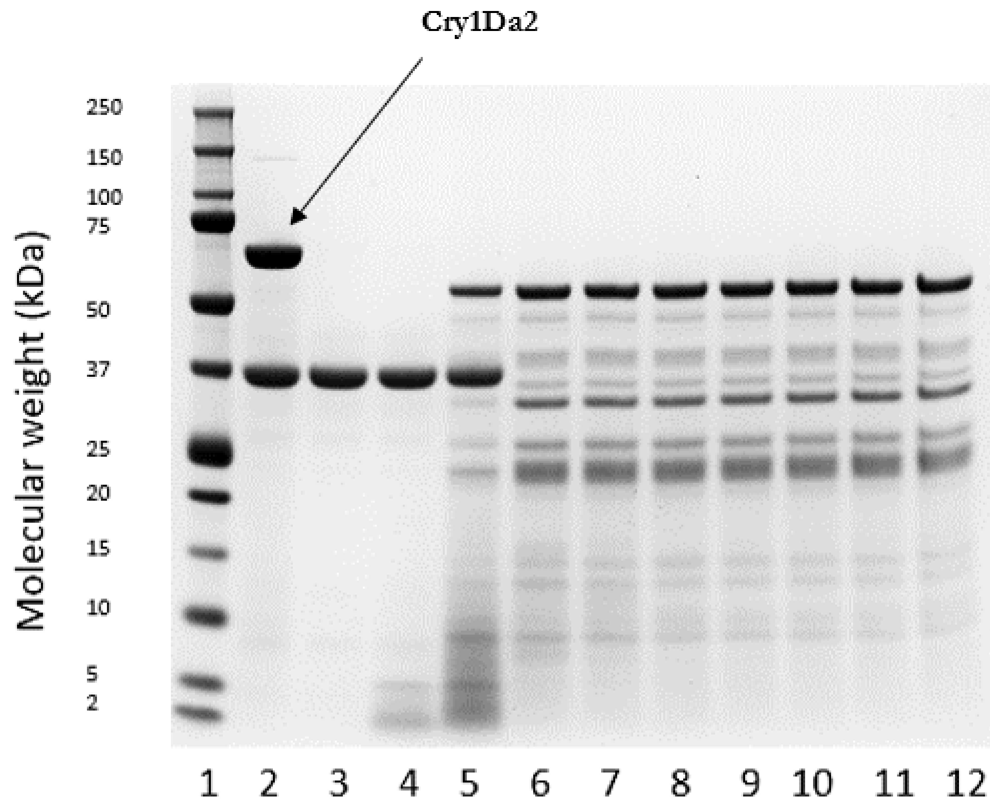
Figure 27. Western Blot Analysis of Cry1Da2 Protein in Simulated Intestinal Fluid Digestion Time Course

Sequential Digestibility Analysis with Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) (PHI-2021-100 study)

Sequential digestion in simulated intestinal fluid (SIF) following a digestion in SGF was used to assess the susceptibility of the low molecular weight SGF fragments (~2-5 kDa, Figure 24) of the Cry1Da2 protein. The Cry1Da2 protein was incubated for 1 minutes in SGF and then incubated for 0, 0.5, 1, 2, 5, 10, 20, and 30 minutes in SIF.

Sequential pepsin and pancreatin digestion results indicated the low molecular weight bands (~2-5 kDa) observed in SGF digestion (Figure 24) were digested within 0.5 minutes during sequential SIF digestion (Figure 28).

Additional details regarding analytical methods are provided in Appendix E.



Lane	Sample Descriptions
1	Pre-stained protein molecular weight marker[a]
2	Cry1Da2 Protein in SGF, Time 0
3	SGF Control, 1 minute
4	Cry1Da2 Protein in SGF, 1 minute
5	Cry1Da2 Protein in SGF 1 minute, SIF Time 0
6	Cry1Da2 Protein in SGF 1 minute, SIF 0.5 minutes
7	Cry1Da2 Protein in SGF 1 minute, SIF 1 minute
8	Cry1Da2 Protein in SGF 1 minute, SIF 2 minutes
9	Cry1Da2 Protein in SGF 1 minute, SIF 5 minutes
10	Cry1Da2 Protein in SGF 1 minute, SIF 10 minutes
11	Cry1Da2 Protein in SGF 1 minute SIF 20 minutes
12	Cry1Da2 Protein in SGF 1 minute, SIF 30 minutes

Note: kilodalton (kDa), simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

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

Figure 28. SDS-PAGE Analysis of Cry1Da2 Protein in a Sequential Digestion with Simulated Gastric Fluid and Simulated Intestinal Fluid

Glycoprotein Analysis (PHI-2021-148 study)

As stated previously in the characterization section, the results from glycoprotein staining analysis confirmed the absence of glycosylation for Cry1Da2 protein isolated and purified from DAS1131 maize tissue.

Evaluation of the Acute Toxicity of Cry1Da2 Protein (PHI-2020-187 study)

A study conducted to evaluate the potential acute toxicity of the test substance, Cry1Da2 protein, in ██████ mice following oral exposure at the limit dose (5000 mg/kg body weight, adjusted for Cry1Da2 content). Cry1Da2 protein and Bovine Serum Albumin (BSA) protein were each reconstituted in deionized water. Vehicle control, BSA comparative control and Cry1Da2 test substance formulations were administered orally by gavage in three split doses, separated by approximately four hours; the BSA comparative control was administered at an equivalent target dose to that of the test substance. The mice were fasted prior to and throughout the dosing procedure.

Body weights were evaluated on test days 1 (prior to fasting and shortly prior to administration of the first dose), 2, 3, 5, 8, and 15. Clinical signs were evaluated ten times on test day 1 (distributed before and after each dose) and daily thereafter. On test day 15, all surviving mice were euthanized and given a gross pathological examination.

All animals survived to scheduled euthanasia. There were no test substance-related clinical observations and all animals gained weight during the 2-week observation period prior to euthanasia. No gross lesions were observed in this study.

Under the conditions of this study, intragastric exposure of Cry1Da2 protein to male and female mice at 5000 mg/kg body weight did not result in mortality or other evidence of acute oral toxicity, based on evaluation of body weight, clinical signs, and gross pathology. Therefore, the acute oral toxicity tolerant dose and the LD_{50} of Cry1Da2 protein was determined to be greater than 5000 mg/kg body weight.

Conclusions on the Safety of Cry1Da2 Protein in DAS1131 Maize

In conclusion, protein characterization results via SDS-PAGE, western blot, glycosylation, mass spectrometry, and N-terminal amino acid sequence analysis have demonstrated that the Cry1Da2 protein derived from DAS1131 maize is of the expected molecular weight, immunoreactivity, lack of glysoylation, and amino acid sequence. Characterization of the microbially derived Cry1Da2 protein demonstrated that it is an appropriate test substance for use in safety studies.

The allergenic and toxic potential of the Cry1Da2 protein was assessed using a bioinformatic comparison of the amino acid sequence of the Cry1Da2 protein to known or putative protein allergen and toxin sequences, evaluation of the stability of the Cry1Da2 protein using *in vitro* gastric and intestinal digestion models, determination of the Cry1Da2 protein glycosylation status, evaluation of the heat lability of the Cry1Da2 protein using a sensitive insect bioassay, and an evaluation of acute toxicity in mice following oral exposure to the Cry1Da2 protein.

The bioinformatic comparisons of the Cry1Da2 protein sequence to known and putative allergen and toxin sequences showed that the Cry1Da2 protein is unlikely to be allergenic or toxic for

humans or animals. The Cry1Da2 protein migrating at ~68 kDa was digested within 0.5 minutes in SGF. The band migrating at ~15 kDa was digested within 5 minutes in SGF and some low molecular weight bands (~2-5 kDa) remained detectable in the Cry1Da2 protein samples for up to 60 minutes in SGF. The protein was digested in SIF, and some bands remained visible after 60 minutes. The low molecular weight bands remaining from SGF digestion were digested (< 0.5 minutes) in sequential SIF. The Cry1Da2 protein heated for approximately 30-35 minutes at targeted temperatures of 75°C or higher was inactive against *S. frugiperda* when incorporated in an artificial diet. The acute oral toxicity assessment determined the LD_{50} of the Cry1Da2 protein to be greater than 5000 mg/kg. These data support the conclusion that DAS1131 maize is as safe as conventional maize for the food and feed supply.

Based on this weight of evidence, consumption of the Cry1Da2 protein from DAS1131 maize is unlikely to cause an adverse effect on humans or animals.

DGT-28 EPSPS Protein

Amino Acid Sequence of the DGT-28 EPSPS Protein

The DGT-28 EPSPS protein is encoded by the *dgt-28* epsps gene derived from *Streptomyces sviceus*. A chimeric chloroplast transit peptide, TraP8, derived from *Brassica napus* and *Brassica rapa* was fused to the N-terminus to target the protein expression in chloroplast. The deduced sequence of the DGT-28 EPSPS precursor protein has 481 amino acids and a molecular weight of approximately 51 kDa (Figure 29).

```
  1   MAQSSRICHG VQNPCVIISN LSKSNQNKSP FSVSLKTHQQ QRRAYQISSW
 51   GLKKSNNGSV IRPVKAARGM PALSLPGSKS ITARALFLAA AADGVTTLVR
101   PLRSDDTEGF AEGLVRLGYR VGRTPDTWQV DGRPQGPAVA EADVYCRDGA
151   TTARFLPTLA AAGHGTYRFD ASPQMRRRPL LPLSRALRDL GVDLRHEEAE
201   GHHPLTVRAA GVEGGEVTLD AGQSSQYLTA LLLLGPLTRQ GLRIRVTDLV
251   SAPYVEITLA MMRAFGVEVA REGDVFVVPP GGYRATTYAI EPDASTASYF
301   FAAAALTPGA EVTVPGLGTG ALQGDLGFVD VLRRMGAEVS VGADATTVRG
351   TGELRGLTAN MRDISDTMPT LAAIAPFASA PVRIEDVANT RVKECDRLEA
401   CAENLRRLGV RVATGPDWIE IHPGPATGAQ VTSYGDHRIV MSFAVTGLRV
451   PGISFDDPGC VRKTFPGFHE AFAELRRGIG S*
```

Figure 29. Deduced Amino Acid Sequence of DGT-28 EPSPS Precursor Protein Encoded by the *dgt-28 epsps* Gene

The deduced amino acid sequence from the translation of the *dgt-28 epsps* gene from plasmid PHP88492 containing the chloroplast transit peptide 8 (TraP8) and the peptide linker. The TraP8 peptide is underlined, and the peptide linker is outlined. The asterisk (*) indicates the translational stop codon. The deduced sequence of the DGT-28 EPSPS precursor protein has 481 amino acids and a molecular weight of approximately 51 kDa which includes the 65-amino acid TraP8 peptide as well as a 2-amino acid linker. The chloroplast transit peptide of the precursor protein is expected to be cleaved upon transport into the chloroplast, resulting in the mature form of DGT-28 EPSPS with a molecular weight of approximately 45 kDa.

Function and Activity of the DGT-28 EPSPS Protein

DGT-28 EPSPS is a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway that catalyzes the reaction of shikimate-3-phosphate (S3P) with phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate 3-phosphate and inorganic phosphate (Griffin *et al.*, 2021). The expressed DGT-28 EPSPS protein is targeted to the maize chloroplasts through the TraP8 peptide to provide tolerance to glyphosate herbicide.

Characterization of the DGT-28 EPSPS Protein Derived from DAS1131 Maize and Microbial System (PHI-2021-151 and PHI-2021-049 studies)

The DGT-28 EPSPS protein expressed in DAS1131 maize was purified from the leaf tissue using ammonium sulfate fractionation and immunoaffinity chromatography.

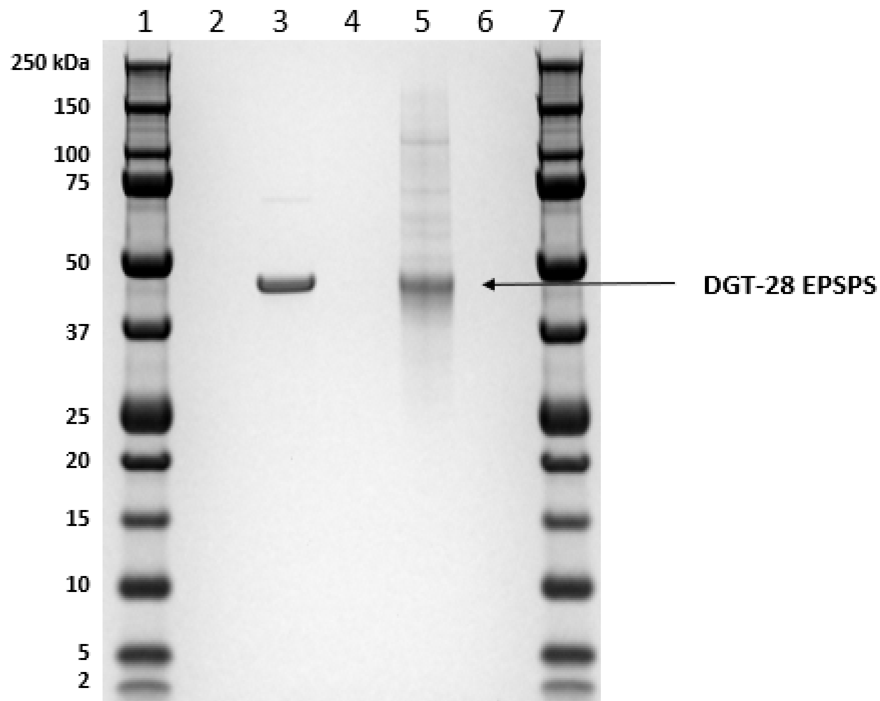
In order to have sufficient amounts of the purified DGT-28 EPSPS protein for the multiple studies required to assess its safety, the large quantity of the DGT-28 EPSPS protein was produced in an *E. coli* protein expression system as a fusion protein with an N-terminal His tag. The His tag was used to facilitate purification of the protein in large quantity using Ni-NTA affinity chromatography. The protein was buffer-exchanged using tangential flow filtration to change the buffer to 25 mM glycine-KOH, 1 mM KH_2PO_4, 0.5 mM EDTA, 25 mM Trehalose, 0.5 mM TCEP, pH 9.5.

The biochemical characteristics of the microbially derived DGT-28 EPSPS protein and the DAS1131 maize-derived DGT-28 EPSPS protein were characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, protein glycosylation analysis, N terminal amino acid sequencing, and peptide mapping by liquid chromatography mass spectrometry (LC-MS). The results demonstrated that the DGT-28 EPSPS protein derived from DAS1131 maize is of the expected molecular weight, immunoreactivity, lack of glysoylation, and amino acid sequence. The microbially derived DGT-28 EPSPS protein was demonstrated to be enzymatically active with the expected molecular weight, immunoreactivity, lack of glysoylation, and amino acid sequence. Characterization of the microbially derived DGT-28 EPSPS protein demonstrated that it is an appropriate test substance for use in safety studies.

SDS-PAGE Analysis

Samples of the purified the maize-derived DGT-28 EPSPS protein and the microbially derived DGT-28 EPSPS protein were analyzed by SDS-PAGE. As expected, the DGT-28 EPSPS proteins migrated as a band consistent with the expected molecular weight of approximately 45 kDa, as shown in Figure 30.

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	Microbially Derived DGT-28 EPSPS Protein (Lot PCF-0054)[b]
4	1X LDS/DTT Sample Buffer Blank
5	DAS1131 Maize-Derived DGT-28 EPSPS Protein[c]
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa).

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

[b] Diluted to 1 µg per lane.

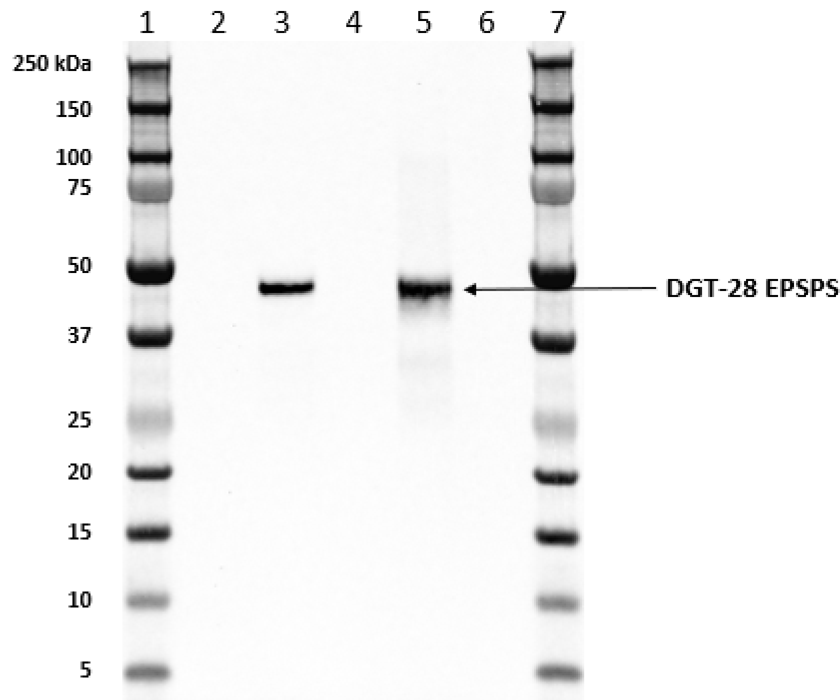
[c] After 1:8 dilution.

Figure 30. SDS-PAGE Analysis of DGT-28 EPSPS Protein

Western Blot Analysis

Samples of the purified maize-derived DGT-28 EPSPS protein and the microbially derived DGT-28 EPSPS protein were analyzed by Western blot. As expected, both of the DGT-28 EPSPS protein samples were immunoreactive to a DGT-28 EPSPS monoclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 45 kDa, as shown in Figure 31.

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	Microbially Derived DGT-28 EPSPS Protein (Lot PCF-0054)[b]
4	1X LDS/DTT Sample Buffer Blank
5	DAS1131 Maize-Derived DGT-28 EPSPS Protein[c]
6	1X LDS/DTT Sample Buffer Blank
7	Pre-stained Protein Molecular Weight Marker[a]

Note: kilodalton (kDa).

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

[b] Diluted to 10 ng per lane.

[c] After 1:800 dilution.

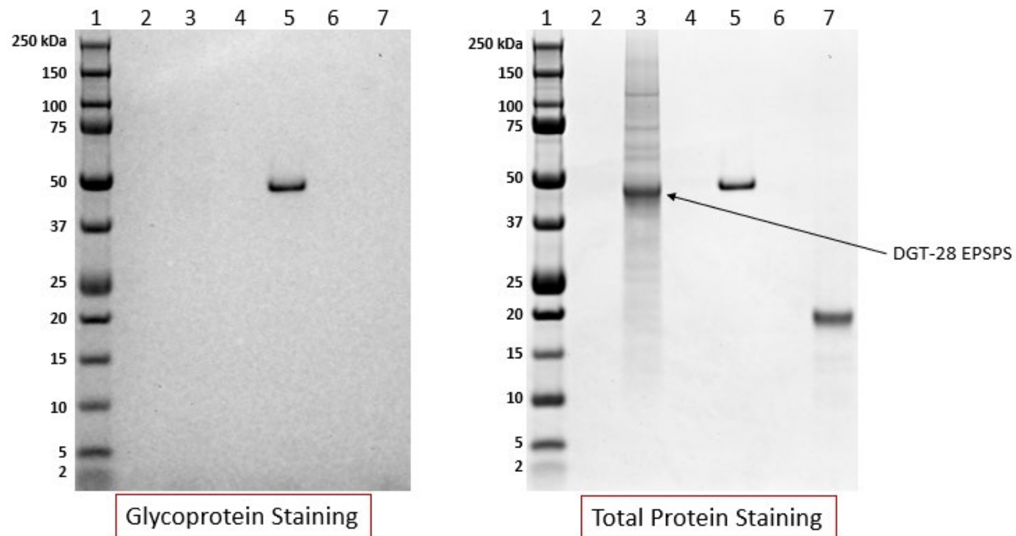
Figure 31. Western Blot Analysis of DGT-28 EPSPS Protein

Protein Glycosylation Analysis

Samples of the purified maize-derived DGT-28 EPSPS protein and the microbially derived DGT-28 EPSPS 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 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 both the DAS1131 maize-derived and microbially derived DGT-28 EPSPS proteins (Figure 32 and Figure 33, respecctively). 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 glycosylation analytical methods are provided in Appendix F.



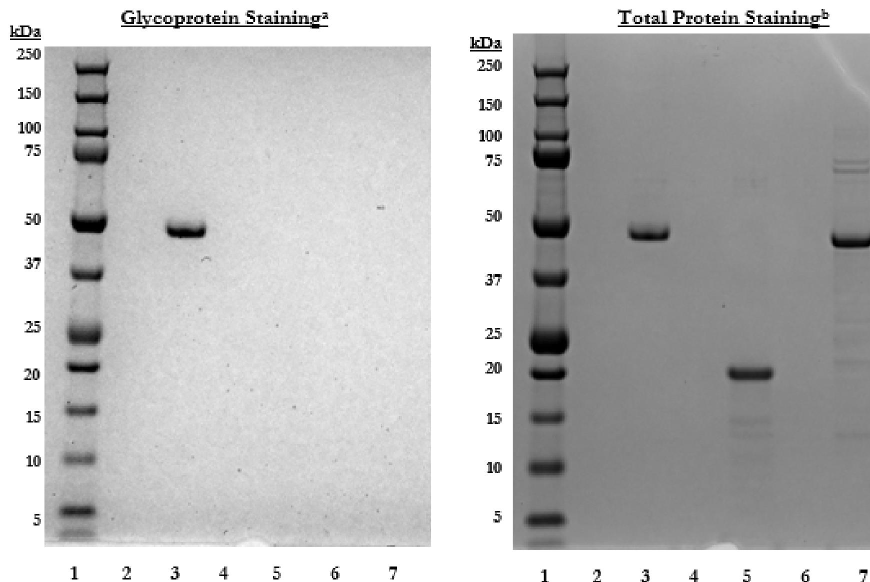
Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS/DTT Sample Buffer Blank
3	DAS1131 Maize-Derived DGT-28 EPSPS Protein[b]
4	1X LDS/DTT Sample Buffer Blank
5	Horseradish Peroxidase (1.0 µg)
6	1X LDS/DTT Sample Buffer Blank
7	Soybean Trypsin Inhibitor (1.0 µg)

Note: The glycoprotein staining gel was stained with glycoprotein staining reagent. The total protein staining gel was stained with glycoprotein staining reagent followed by staining with Coomassie blue reagent for total proteins. Kilodalton (kDa) and microgram (µg).

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

[b] After 1:4 dilution.

Figure 32. Glycosylation Analysis of DAS1131 Maize-Derived DGT-28 EPSPS Protein



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[c]
2	1X LDS/DTT Sample Buffer Blank
3	Horseradish Peroxidase Positive Control (1 µg)
4	1X LDS/DTT Sample Buffer Blank
5	Soybean Trypsin Inhibitor Negative Control (1 µg)
6	1X LDS Sample Buffer Blank
7	Microbially Derived DGT-28 EPSPS Protein (1 µg)

Note: kilodalton (kDa) and microgram (µg).

[a] Gel was stained with glycoprotein staining reagent.

[b] Gel was stained with glycoprotein staining reagent followed by staining with Coomassie Blue Reagent for total proteins.

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

Figure 33. Glycosylation Analysis of Microbially Derived DGT-28 EPSPS Protein

Mass Spectrometry Peptide Mapping Analysis

Samples of the purified maize-derived DGT-28 EPSPS protein and the microbially derived DGT-28 EPSPS protein were analyzed by SDS-PAGE. Protein bands were stained with Coomassie stain reagent, and bands containing the DGT-28 EPSPS protein were excised for each sample.

The excised protein bands containing the partially purified maize-derived DGT-28 EPSPS protein were digested with chymotrypsin. The excised protein bands containing the microbially derived DGT-28 EPSPS protein were digested with trypsin and chymotrypsin. Digested samples were analyzed with ultra-performance liquid chromatography-mass spectrometry (LC-MS). The resulting MS data were used to search and match the peptides from the expected DGT-28 EPSPS protein sequence, and the sequence coverage was calculated.

The LC-MS analysis of the chymotrypsin-digested DAS1131 maize-derived DGT-28 EPSPS protein identified 52 peptides including two identified N-terminal peptide sequences resulting in 86% coverage of the DGT-28 EPSPS protein sequence of the mature protein (Table 22, Figure 34).

The identified tryptic and chymotryptic peptides for the microbially derived DGT-28 EPSPS protein are shown in Table 23 and Table 24, respectively. The combined sequence coverage for the microbially derived DGT-28 EPSPS protein was 81.0% (342/422) of the expected DGT-28 EPSPS amino acid sequence (Table 25 and Figure 35).

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

Table 22. Identified Chymotryptic Peptides of DAS1131 Maize-Derived DGT-28 EPSPS Protein Using LC-MS Analysis

Matched Residue Position (A Form)	Matched Residue Position (AA Form)	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
1–20	NA	1996 1383	1996 1251	ARGMPALSLPGSKSITARAL
1–21	NA	2143 2076	2143 1935	ARGMPALSLPGSKSITARALF
NA	1–21	2067 1759	2067 1622	AARGMPALSLPGSKSITARAL
NA	1–22	2214 2463	2214 2307	AARGMPALSLPGSKSITARALF
8–20	9–21	1299 7569	1299 751	SLPGSKSITARAL
8–21	9–22	1446 8275	1446 8194	SLPGSKSITARALF
21–32	22–33	1148 6136	1148 6077	FLAAAADGVTTL
22–36	23–37	1466 8557	1466 8457	LAAAADGVTTLVRPL
33–44	34–45	1390 6913	1390 6841	VRPLRSDDTEGF
33–48	34–49	1760 8815	1760 8693	VRPLRSDDTEGFAEGL
37–48	38–49	1295 5696	1295 563	RSDDTEGFAEGL
37–51	38–52	1663 8282	1663 8166	RSDDTEGFAEGLVRL
45–51	46–52	756 4527	756 4494	AEGLVRL
45–53	46–54	976 5388	976 5342	AEGLVRLGY
54–62	55–63	1086 5622	1086 5571	RVGRTPDTW
54–79	55–80	2839 4252	2839 4002	RVGRTPDTWQVDGRPQGPAVAEADVY
63–79	64–80	1770 8619	1770 8537	QVDGRPQGPAVAEADVY
80–89	81–90	1153 5332	1153 5298	CRDGATTARF
80–101	81–102	2306 1389	2306 1226	CRDGATTARFLPTLAAAGHGTY
90–101	91–102	1170 6085	1170 6033	LPTLAAAGHGTY
115–121	116–122	768 4898	768 4857	LPLSRAL
122–128	123–129	786 4274	786 4236	RDLGVDL
122–139	123–140	2079 0362	2079 0246	RDLGVDLRHEEAEGHHPL
125–139	126–140	1694 8202	1694 8125	GVDLRHEEAEGHHPL
129–139	130–140	1310 616	1310 6116	RHEEAEGHHPL
140–153	141–154	1357 7277	1357 7202	TVRAAGVEGGEVTL
140–161	141–162	2194 062	2194 0502	TVRAAGVEGGEVTLDAGQSSQY
140–162	141–163	2307 1531	2307 1343	TVRAAGVEGGEVTLDAGQSSQYL
168–176	169–177	953 5706	953 5658	LGPLTRQGL
169–176	170–177	840 4848	840 4818	GPLTRQGL
177–188	178–189	1388 7855	1388 7776	RIRVTDLVSAPY
189–199	190–200	1312 6592	1312 6519	VEITLAMMRAF [c]
194–199	195–200	725 3381	725 3353	AMMRAF
200–210	201–211	1176 5828	1176 5775	GVEVAREGDVF
223–233	224–234	1123 5074	1123 5033	AIEPDASTASY
223–234	224–235	1270 5792	1270 5717	AIEPDASTASYF
236–256	237–257	1836 0123	1835 9993	AAAALTPGAEVTVPGLGTGAL
241–256	242–257	1438 7774	1438 7668	TPGAEVTVPGLGTGAL
267–288	268–289	2232 1421	2232 1281	RRMGAEVSVGADATTVRGTGEL
267–291	268–292	2558 3513	2558 3347	RRMGAEVSVGADATTVRGTGELRGL
289–305	290–306	1890 9418	1890 9292	RGLTANMRDISDTMPTL
289–311	290–312	2461 2662	2461 2457	RGLTANMRDISDTMPTLAAIAPF
292–305	293–306	1564 7326	1564 7225	TANMRDISDTMPTL
292–311	293–312	2135 0492	2135 0391	TANMRDISDTMPTLAAIAPF
312–332	313–333	2398 2513	2398 2387	ASAPVRIEDVANTRVKECDRL
312–339	313–340	3185 5818	3185 5557	ASAPVRIEDVANTRVKECDRLEACAENL
333–342	334–343	1230 621	1230 6139	EACAENLRRL
343–368	344–369	2678 3677	2678 3453	GVRVATGPDWIEIHPGPATGAQVTSY
353–368	354–369	1639 8335	1639 8206	IEIHPGPATGAQVTSY
378–389	379–390	1215 7046	1215 6976	AVTGLRVPGISF
383–389	384–390	774 4425	774 4388	RVPGISF
383–406	384–407	2735 352	2735 3279	RVPGISFDDPGCVRKTFPGFHEAF

Note: In DAS1131 maize, the DGT-28 EPSPS precursor protein contains a chloroplast transit peptide (CTP) which is cleaved upon transport into the chloroplast, resulting in the mature form of the protein. The CTP of the DGT-28 EPSPS was cleaved at two alternative positions (A66 or A67), resulting in two identified N-termini, one beginning with A (A Form) and the other AA (AA Form). Not applicable (NA); this peptide was not identified for this form.

[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 closest to the experimental mass.

[c] This peptide was modified by methionine oxidation.

```
  1 MAQSSRICHG VQNPCVIISN LSKSNQNKSP FSVSLKTHQQ QRRAYQISSW
 51 GLKKSNNGSV IRPVKAARGM PALSLPGSKS ITARALFLAA AADGVTTLVR
101 PLRSDDTEGF AEGLVRLGYR VGRTPDTWQV DGRPQGPAVA EADVYCRDGA
151 TTARFLPTLA AAGHGTYRFD ASPQMRRRPL LPLSRALRDL GVDLRHEEAE
201 GHHPLTVRAA GVEGGEVTLD AGQSSQYLTA LLLLGPLTRQ GLRIRVTDLV
251 SAPYVEITLA MMRAFGVEVA REGDVFVVPP GGYRATTYAI EPDASTASYF
301 FAAAALTPGA EVTVPGLGTG ALQGDLGFVD VLRRMGAEVS VGADATTVRG
351 TGELRGLTAN MRDISDTMPT LAAIAPFASA PVRIEDVANT RVKECDRLEA
401 CAENLRRLGV RVATGPDWIE IHPGPATGAQ VTSYGDHRIV MSFAVTGLRV
451 PGISFDDPGC VRKTFPGFHE AFAELRRGIG S
```

Red type	Bold red type indicates DAS1131 maize-derived DGT-28 EPSPS peptides identified using LC-MS analysis. The CTP is underlined, and the peptide linker is outlined in a box.
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: In DAS1131 maize, the DGT-28 EPSPS precursor protein contains a chloroplast transit peptide (CTP) which is cleaved upon transport into the chloroplast, resulting in the mature form of the protein. The CTP of the DGT-28 EPSPS was cleaved at two alternative positions (A66 or A67), resulting in two identified N-termini, one beginning with A and the other with AA.

Figure 34. Identified Chymotryptic Peptide Amino Acid Sequence of DAS1131 Maize-Derived DGT-28 EPSPS Protein Using LC-MS Analysis

Table 23. Identified Tryptic Peptides of Microbially Derived DGT-28 EPSPS Protein Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
10–20	1056 5537	1056 5638	GMPALSLPGSK
45–57	1394 6165	1394 6314	SDDTEGFAEGLVR
65–88	2687 2169	2687 2398	TPDTWQVDGRPQGPAVAEADVYCR
96–109	1473 7601	1473 7728	FLPTLAAAGHGTYR
110–117	950 4191	950 428	FDASPQMR
118–126	1106 697	1106 7036	RRPLLPLSR
119–126	950 5937	950 6025	RPLLPLSR
130–136	786 4178	786 4236	DLGVDLR
205–212	847 4485	847 4552	AFGVEVAR
213–225	1390 6742	1390 6881	EGDVFVVPPGGYR
275–290	1618 794	1618 8097	RMGAEVSVGADATTVR
276–290	1462 6931	1462 7086	MGAEVSVGADATTVR
291–296	631 3254	631 3289	GTGELR
297–303	761 3799	761 3854	GLTANMR
304–324	2143 0775	2143 0983	DISDTMPTLAAIAPFASAPVR
325–332	916 4536	916 4614	IEDVANTR
333–338	805 3689	805 3752	VKECDR
335–347	1634 6975	1634 714	ECDRLEACAENLR
339–347	1074 5019	1074 5128	LEACAENLR
339–348	1230 6058	1230 6139	LEACAENLRR
353–379	2831 3387	2831 3627	VATGPDWIEIHPGPATGAQVTSYGDHR
380–390	1208 6461	1208 6587	IVMSFAVTGLR[c]
391–403	1417 6508	1417 666	VPGISFDDPGCVR
391–404	1545 7446	1545 761	VPGISFDDPGCVRK
405–417	1520 7275	1520 7412	TFPGFHEAFAELR

[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 closest to the experimental mass.
[c] This peptide was modified by methionine oxidation (Oxidation-M).

Table 24. Identified Chymotryptic Peptides of Microbially Derived DGT-28 EPSPS Protein Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
15–27	1299 7416	1299 751	SLPGSKSITARAL
28–39	1148 5955	1148 6077	FLAAAADGVTTL
29–39	1001 5288	1001 5393	LAAAADGVTTL
44–55	1295 55	1295 563	RSDDTEGFAEGL
44–58	1663 8	1663 8166	RSDDTEGFAEGLVRL
52–60	976 5252	976 5342	AEGLVRLGY
70–86	1770 8332	1770 8537	QVDGRPQGPAVAEADVY
87–96	1153 5235	1153 5298	CRDGATTARF
97–108	1170 5912	1170 6033	LPTLAAAGHGTY
101–108	746 3291	746 3347	AAAGHGTY
122–128	768 4796	768 4857	LPLSRAL
129–135	786 4181	786 4236	RDLGVDL
129–146	2079 0123	2079 0246	RDLGVDLRHEEAEGHHPL
147–160	1357 7044	1357 7202	TVRAAGVEGGEVTL
147–168	2194 0239	2194 0502	TVRAAGVEGGEVTLDAGQSSQY
147–169	2307 1115	2307 1343	TVRAAGVEGGEVTLDAGQSSQYL
161–169	967 4157	967 4247	DAGQSSQYL
175–183	953 5573	953 5658	LGPLTRQGL
176–183	840 4745	840 4818	GPLTRQGL
196–206	1312 6369	1312 6519	VEITLAMMRAF[c]
201–206	741 3245	741 3302	AMMRAF[c]
207–217	1176 5655	1176 5775	GVEVAREGDV
230–240	1123 4925	1123 5033	AIEPDASTASY
230–241	1270 5579	1270 5717	AIEPDASTASYF
299–312	1564 7061	1564 7225	TANMRDISDTMPTL
299–318	2167 0042	2167 0289	TANMRDISDTMPTLAAIAPF[c]
319–339	2398 2137	2398 2387	ASAPVRIEDVANTRVKECDRL
376–384	1076 4957	1076 5073	GDHRIVMSF[c]

[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 closest to the experimental mass.
[c] This peptide was modified by methionine oxidation (Oxidation-M).

Table 25. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of Microbially Derived DGT-28 EPSPS Protein Using LC-MS Analysis

Protease	% Coverage	Combined % Coverage
Trypsin	58	81.0
Chymotrypsin	52	

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  1 MHHHHHHARG MPALSLPGSK SITARALFLA AAADGVTTLV RPLRSDDTEG
 51 FAEGLVRLGY RVGRTPDTWQ VDGRPQGPAV AEADVYCRDG ATTARFLPTL
101 AAAGHGTYRF DASPQMRRRP LLPLSRALRD LGVDLRHEEA EGHHPLTVRA
151 AGVEGGEVTL DAGQSSQYLT ALLLLGPLTR QGLRIRVTDL VSAPYVEITL
201 AMMRAFGVEV AREGDVFVVP PGGYRATTYA IEPDASTASY FFAAAALTPG
251 AEVTVPGLGT GALQGDLGFV DVLRRMGAEV SVGADATTVR GTGELRGLTA
301 NMRDISDTMP TLAAIAPFAS APVRIEDVAN TRVKECDRLE ACAENLRRLG
351 VRVATGPDWI EIHPGPATGA QVTSYGDHRI VMSFAVTGLR VPGISFDDPG
401 CVRKTFPGFH EAFAELRRGI GS
```

Red bold type	Red type indicates microbially derived DGT-28 EPSPS peptides identified using LC-MS analysis against the expected[a] microbially derived DGT-28 EPSPS sequence.
Amino acid residue abbreviations	alanine (A), cysteine (C), 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).

[a] The expected sequence refers to the translated sequence for the gene derived from the expression vector and used for production of the DGT-28 EPSPS protein toxicology lot.

Figure 35. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of Microbially Derived DGT-28 EPSPS Protein Using LC-MS Analysis

N-Terminal Amino Acid Sequence Analysis

The Edman sequencing analysis of the purified maize-derived DGT-28 EPSPS protein sample identified two sequences (AARGMPALSL and ARGMPALSLP), consistent with the N-terminal chymotryptic peptides identified by LC-MS (Table 26). The N-terminal sequencing and LC-MS analysis of the chymotryptic peptides indicate the chloroplast transit peptide (CTP) of the DGT-28 EPSPS precursor protein was cleaved at two adjacent positions (before amino acid 66 or 67, Figure 34). This is consistent with the observation that CTP cleavage can occur at multiple positions for some nucleus-encoded chloroplast proteins as described by Rowland *et al.* (2015).

The analysis of the microbially derived DGT-28 EPSPS protein using Edman sequencing identified an N-terminal sequence (MHHHHHHARGMPALS), matching amino acid residues 1-15 of the expected sequence (Table 26).

Additional details regarding N-terminal amino acid sequence analytical methods are provided in Appendix F.

Table 26. N-Terminal Amino Acid Sequence Analysis of DAS1131 Maize Derived and Microbially Derived DGT-28 EPSPS Proteins

Description		Amino Acid Sequence
DAS1131 maize derived DGT-28 EPSPS protein	Identified Sequence	A-R-G-M-P-A-L-S-L
	Identified Sequence	A-A-R-G-M-P-A-L-S-L
Microbially derived DGT-28 EPSPS protein	Expected Sequence	M-H-H-H-H-H-H-A-R-G-M-P-A-L-S
	Identified Sequence	M-H-H-H-H-H-H-A-R-G-M-P-A-L-S

Note: In DAS1131 maize, the DGT-28 EPSPS precursor protein contains a chloroplast transit peptide (CTP) which is cleaved upon transport into the chloroplast, resulting in the mature form of the protein. The CTP of the DGT-28 EPSPS was cleaved at two alternative positions (A66 or A67), resulting in two identified N-termini, one beginning with A and the other with AA. The microbially derived DGT-28 EPSPS has an N-terminal His tag to facilitate large scale protein production.

Additional details regarding N-terminal amino acid sequence analytical methods are provided in Appendix F.

Allergenicity and Toxicity Analyses of the DGT-28 EPSPS Protein

A weight-of-evidence approach was applied to determine the allergenic and toxic potential of the DGT-28 EPSPS protein expressed in DAS1131 maize, including an assessment of the following: a bioinformatic comparison of the amino acid sequence of DGT-28 EPSPS protein to known or putative protein allergen and toxin sequences, evaluation of the stability of the DGT-28 EPSPS protein using *in vitro* gastric and intestinal digestion models, determination of the DGT-28 EPSPS protein glycosylation status, evaluation of the heat lability of the DGT-28 EPSPS protein using a enzymic assay , and an evaluation of acute toxicity in mice following oral exposure to DGT-28 EPSPS protein (Carlson *et al.*, 2019).

Bioinformatic Analysis of Homology to Known or Putative Allergens (PHI-2021-074/201 study)

Assessing newly expressed proteins for potential cross-reactivity with known or putative allergens is an important part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically-modified plant products (Codex Alimentarius Commission, 2003). A bioinformatic assessment of the DGT-28 EPSPS protein sequence for potential cross-reactivity with known or putative allergens was conducted according to relevant guidelines (Codex Alimentarius Commission, 2003; FAO/WHO, 2001).

Two separate searches for the DGT-28 EPSPS protein sequence were performed using the Comprehensive Protein Allergen Resource (COMPARE) 2021 database (January 2021) available at http://comparedatabase.org. This peer-reviewed database is a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins, and Bioinformatics (PATB) Committee and is comprised of 2,348 sequences. The first search used the DGT-28 EPSPS protein sequence as the query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the allergen sequences. The search was conducted using default parameters, except the *E*-score threshold was set to 10^{-4}. An *E*-score threshold of 10^{-4} has been shown to be an appropriate value for allergenicity searches (Mirsky *et al.*, 2013). The generated alignments were examined to identify any that are 80 residues or longer and possess a sequence identity of greater than 35%. The second search used the FUZZPRO program (Emboss Package v6.4.0) to identify any contiguous 8-residue identical matches between the DGT-28 EPSPS protein sequence and the allergen sequences.

Results of the search of the DGT-28 EPSPS protein sequence against the COMPARE database of known and putative allergens found no alignments that were 80 residues or longer with a sequence identity of greater than 35%. No contiguous 8-residue matches between the DGT-28 EPSPS protein sequence and the allergen sequences were identified in the second search.

Collectively, these data indicate that no allergenicity concern arose from the bioinformatics assessment of the DGT-28 EPSPS protein.

Bioinformatic Analysis of Homology to Known or Putative Toxins (PHI-2021-209/211 study)

Assessing newly expressed proteins for potential toxicity is a critical part of the weight-of-evidence approach used to evaluate the safety of these proteins in genetically modified plant products (Codex Alimentarius Commission, 2003). The potential toxicity of the 5-enolpyruvylshikimate-3-phosphate synthase (DGT-28 EPSPS) protein was assessed by

comparison of its sequence to the sequences in an internal toxin database. The internal toxin database is a subset of sequences found in UniProtKB/Swiss-Prot (https://www.uniprot.org/). To produce the internal toxin database, the manually annotated proteins in UniProtKB/Swiss-Prot are filtered for molecular function by keywords that could imply toxicity or adverse health effects (e.g., toxin, hemagglutinin, vasoactive, etc.). The internal toxin database is updated annually. The search between the DGT-28 EPSPS protein sequence and protein sequences in the internal toxin database was conducted with BLASTP using default parameters, except that low complexity filtering was turned off, the *E*-value threshold was set to 10^{-4}, and unlimited alignments were returned.

No alignments with an *E*-value $\leq 10^{-4}$ were returned between the DGT-28 EPSPS protein sequence and any protein sequence in the internal toxin database. Therefore, no toxicity concerns arose from the bioinformatics assessment of the DGT-28 EPSPS protein.

Thermolability Analysis (PHI-2021-145 study)

Thermal stability of the DGT-28 EPSPS protein was characterized by assessing enzyme activity after heating at 25 °C, 37 °C, 50 °C, or 75 °C for 30-35 minutes. DGT-28 EPSPS is a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway that catalyzes the reaction of shikimate-3-phosphate (S3P) with phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate 3-phosphate and inorganic phosphate. DGT-28 EPSPS activity was determined by measuring the quantity of free phosphate released as a product by the reaction of S3P and PEP. The activity for each sample was determined using a 3:1 ratio of malachite green solution and ammonium molybdate solution and absorbance was read at 660 nm. Activity in the aliquots heat-treated at 25 °C, 37 °C, 50 °C, or 75 °C was compared to activity in the unheated control.

The results demonstrated that the DGT-28 EPSPS protein was inactivated when heat-treated for 30-35 minutes at 50 °C and 75 °C. In addition, the DGT-28 EPSPS protein showed substantially reduced activity when heat-treated for the same length of time at 37 °C (33.4% activity compared to the unheated control). No reduction in activity was observed for DGT-28 EPSPS protein when heat-treated at 25 °C (Table 27).

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

Table 27. Effect of Heat Treatment on DGT-28 EPSPS Protein Enzymatic Activity

Treatment	Mean DGT-28 EPSPS Interpolated Value (µM)	$P0_4$ (µmol/min)	% Activity of Control
Unheated Control Solution	67.119	0.000223731	--
Test Solution Heated to 25 °C	73.829	0.000246098	110.0
Test Solution Heated to 37 °C	22.419	0.00007473	33.4
Test Solution Heated to 50 °C	0	0	0
Test Solution Heated to 75 °C	0	0	0

Note: All treatments were prepared to a target concentration of 1 mg DGT-28 EPSPS protein/ml prior to heat treatment. The unheated control solution was maintained chilled (2-8 °C or on wet ice).

Digestibility Analysis with Simulated Gastric Fluid (PHI-2021-099 study)

Simulated gastric fluid (SGF) containing pepsin at pH ~1.2 was used to assess the susceptibility of the DGT-28 EPSPS protein to proteolytic digestion by pepsin *in vitro*. The DGT-28 EPSPS protein was incubated in SGF for 0, 0.5, 1, 2, 5, 10, 20, and 60 minutes. A positive control (bovine serum albumin) and a negative control (β-lactoglobulin) were included in the assay and were incubated in SGF for 0, 1, and 60 minutes. After incubation in SGF, the samples were analyzed by SDS-PAGE. Coomassie-based stain or western blot was used to detect protein bands.

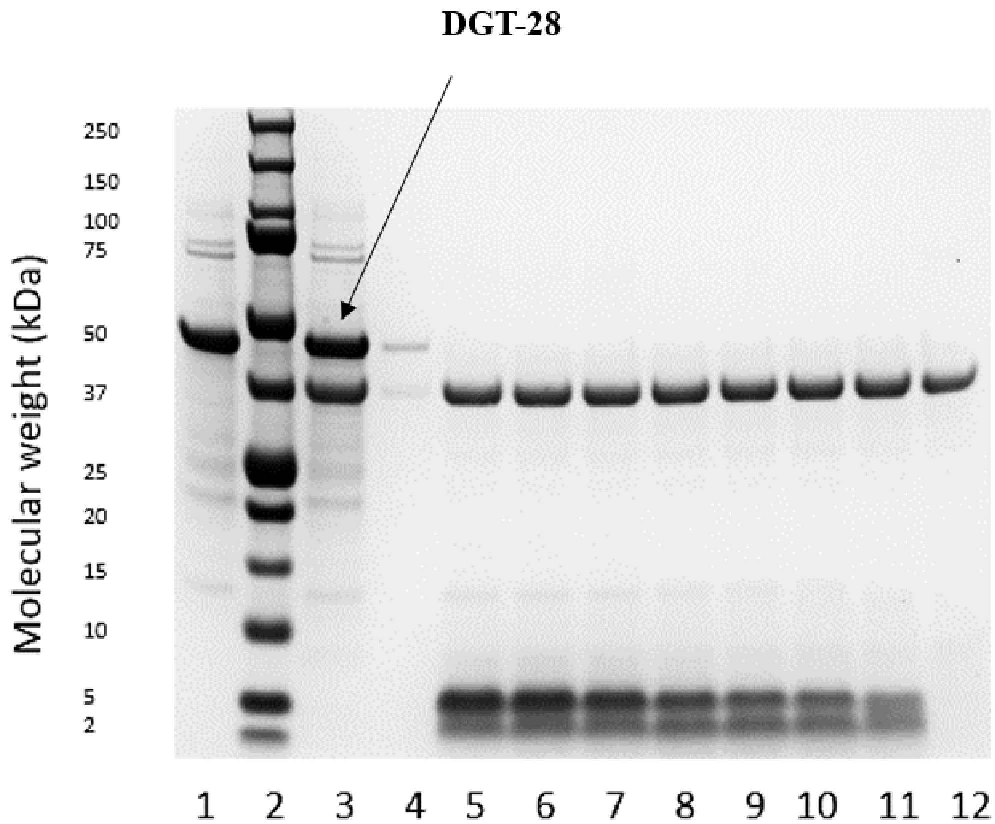
A summary of the SGF results is provided in Table 28. The DGT-28 EPSPS protein migrating at ~45 kDa was digested (within 0.5 minutes) in SGF as demonstrated by both SDS-PAGE and western blot analysis (Figure 36 and Figure 37, respectively). Some low molecular weight bands (~2 and ~5 kDa) on the SDS-PAGE gel remained detectable in the DGT-28 EPSPS protein samples for up to 60 minutes in SGF. As expected, the BSA control was digested in 1 minute and low molecular weight bands remained detectable at 60 minutes. The β-lactoglobulin control remained detectable after 60 minutes in SGF verifying that the assay performed as expected.

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

Table 28. Summary of DGT-28 EPSPS Protein In Vitro Pepsin Resistance Analyses

Protein	Approximate Molecular Weight (kDa[a])	Digestion Time Determined by SDS-PAGE[a] (minutes)	Digestion Time Determined by Western Blot (minutes)
DGT-28 EPSPS	45	≤ 0.5	≤ 0.5
Bovine Serum Albumin	66	≤ 1	NA
β-lactoglobulin	18	> 60	NA

Note: kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and not applicable (NA).

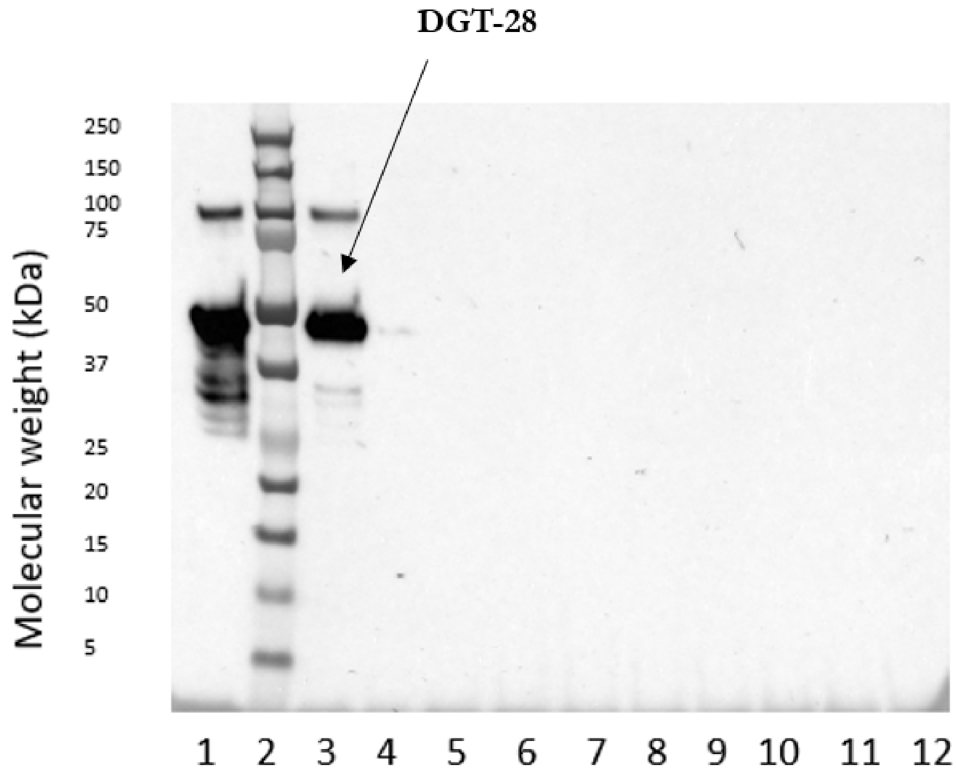


Lane	Sample Descriptions
1	DGT-28 EPSPS protein in water, Time 0
2	Pre-stained protein molecular weight marker[a]
3	DGT-28 EPSPS protein in SGF, Time 0
4	DGT-28 EPSPS protein in SGF, Time 0; 1:20 dilution
5	DGT-28 EPSPS protein in SGF, 0.5 minutes
6	DGT-28 EPSPS protein in SGF, 1 minute
7	DGT-28 EPSPS protein in SGF, 2 minutes
8	DGT-28 EPSPS protein in SGF, 5 minutes
9	DGT-28 EPSPS protein in SGF, 10 minutes
10	DGT-28 EPSPS protein in SGF, 20 minutes
11	DGT-28 EPSPS protein in SGF, 60 minutes
12	SGF Control, 60 minutes

Note: kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and simulated gastric fluid (SGF).

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

Figure 36. SDS-PAGE Analysis of DGT-28 EPSPS Protein in Simulated Gastric Fluid Digestion Time Course



Lane	Sample Descriptions
1	DGT-28 EPSPS protein in water, Time 0
2	Pre-stained protein molecular weight marker[a]
3	DGT-28 EPSPS protein in SGF, Time 0
4	DGT-28 EPSPS protein in SGF, Time 0; 1:200 dilution
5	DGT-28 EPSPS protein in SGF, 0.5 minutes
6	DGT-28 EPSPS protein in SGF, 1 minute
7	DGT-28 EPSPS protein in SGF, 2 minutes
8	DGT-28 EPSPS protein in SGF, 5 minutes
9	DGT-28 EPSPS protein in SGF, 10 minutes
10	DGT-28 EPSPS protein in SGF, 20 minutes
11	DGT-28 EPSPS protein in SGF, 60 minutes
12	SGF Control, 60 minutes

Note: kilodalton (kDa) and simulated gastric fluid (SGF).

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

Figure 37. Western Blot Analysis of DGT-28 EPSPS Protein in Simulated Gastric Fluid Digestion Time Course

Digestibility Analysis with Simulated Intestinal Fluid (PHI-2021-103 study)

Simulated intestinal fluid (SIF) containing pancreatin at ~pH 7.5 was used to assess the susceptibility of the DGT-28 EPSPS protein to proteolytic digestion by pancreatin *in vitro*. The DGT-28 EPSPS protein was incubated in SIF for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 minutes. A positive control (β-lactoglobulin) and a negative control (bovine serum albumin) were included in the assay and were incubated in SIF for 0, 1, and 60 minutes. After incubation in SIF, the samples were analyzed by SDS-PAGE. Coomassie-based stain or western blot was used to detect protein bands.

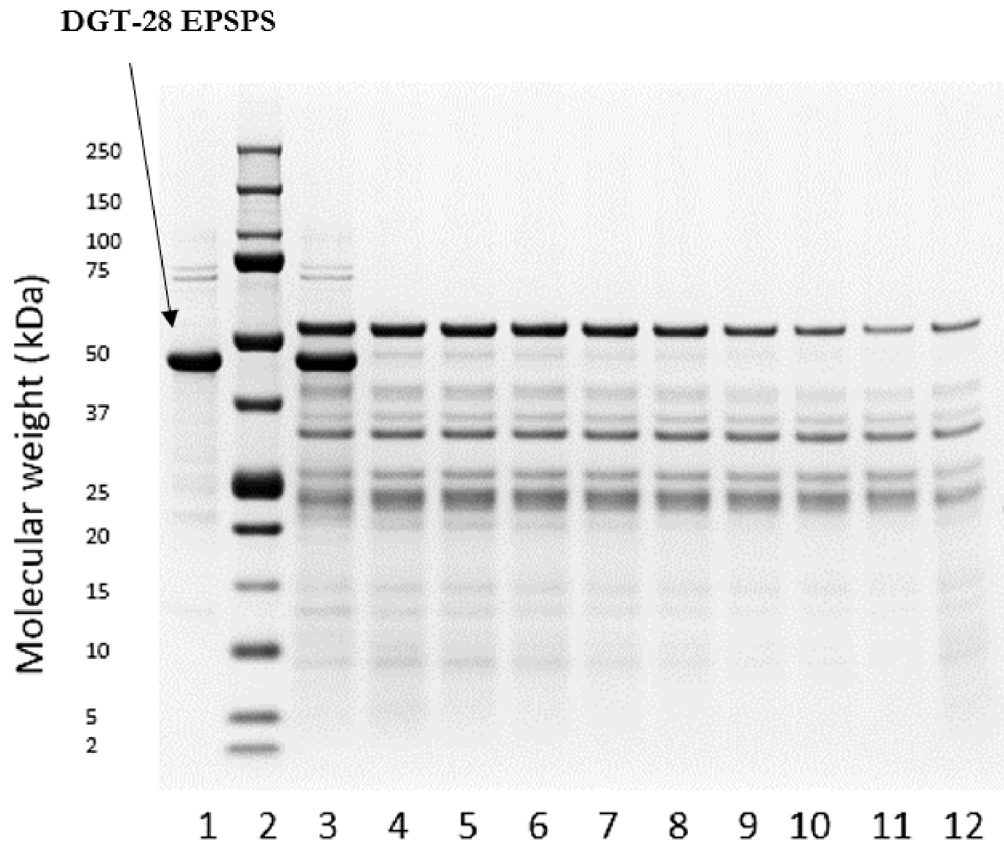
A summary of the SIF assay results is provided in Table 29. The DGT-28 EPSPS protein migrating at ~45 kDa was digested within 0.5 minutes in SIF as demonstrated by both SDS-PAGE and western blot analysis (Figure 38 and Figure 39, respectively). The β-lactoglobulin control was digested within 60 minutes in SIF and low molecular weight bands remained detectable at 60 minutes. The BSA control remained detectable after 60 minutes in SIF, verifying that the assay performed as expected.

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

Table 29. Summary of DGT-28 EPSPS Protein In Vitro Pancreatin Resistance Assay Results

Protein	Approximate MolecularWeight (kDa)	Digestion Time Determined by SDS-PAGE (minutes)	Digestion Time Determined by Western Blot (minutes)
DGT-28 EPSPS	45	≤ 0.5	≤ 0.5
β-Lactoglobulin	18	< 60	NA
Bovine Serum Albumin	66	> 60	NA

Note: kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and not applicable (NA).

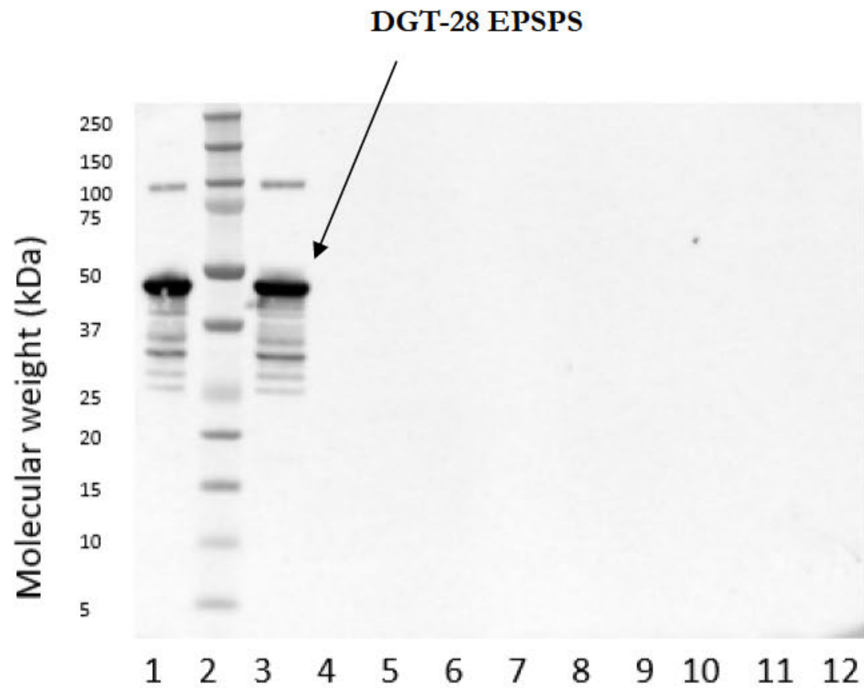


Lane	Sample Descriptions
1	DGT-28 EPSPS protein in water, Time 0
2	Pre-stained protein molecular weight marker[a]
3	DGT-28 EPSPS protein in SIF, Time 0
4	DGT-28 EPSPS protein in SIF, 0.5 minutes
5	DGT-28 EPSPS protein in SIF, 1 minute
6	DGT-28 EPSPS protein in SIF, 2 minutes
7	DGT-28 EPSPS protein in SIF, 5 minutes
8	DGT-28 EPSPS protein in SIF, 10 minutes
9	DGT-28 EPSPS protein in SIF, 20 minutes
10	DGT-28 EPSPS protein in SIF, 30 minutes
11	DGT-28 EPSPS protein in SIF, 60 minutes
12	SIF Control, 60 minutes

Note: kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and simulated intestinal fluid (SIF).

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

Figure 38. SDS-PAGE Analysis of DGT-28 EPSPS Protein in Simulated Intestinal Fluid Digestion Time Course



Lane	Sample Descriptions
1	DGT-28 EPSPS protein in water
2	Pre-stained protein molecular weight marker[a]
3	DGT-28 EPSPS protein in SIF, Time 0
4	DGT-28 EPSPS protein in SIF, 0.5 minutes
5	DGT-28 EPSPS protein in SIF, 1 minute
6	DGT-28 EPSPS protein in SIF, 2 minutes
7	DGT-28 EPSPS protein in SIF, 5 minutes
8	DGT-28 EPSPS protein in SIF, 10 minutes
9	DGT-28 EPSPS protein in SIF, 20 minutes
10	DGT-28 EPSPS protein in SIF, 30 minutes
11	DGT-28 EPSPS protein in SIF, 60 minutes
12	SIF Control, 60 minutes

Note: kilodalton (kDa) and simulated intestinal fluid (SIF).

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

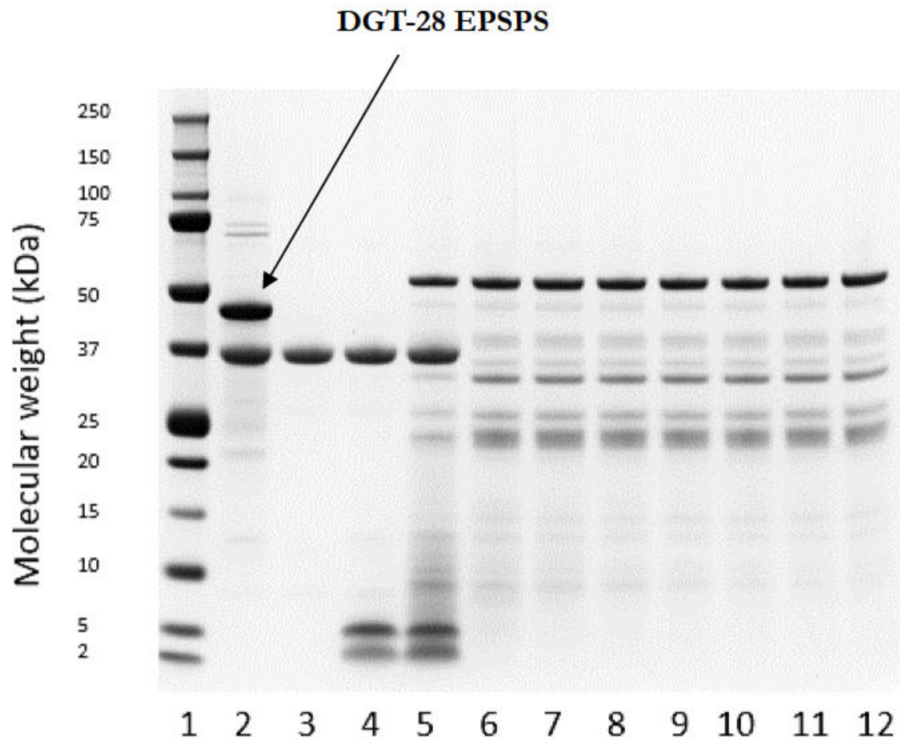
Figure 39. Western Blot Analysis of DGT-28 EPSPS Protein in Simulated Intestinal Fluid Digestion Time Course

Sequential Digestibility Analysis with Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) (PHI-2021-102 study)

Sequential digestion in simulated intestinal fluid (SIF) following a digestion in SGF was used to assess the susceptibility of the low molecular weight SGF fragments (~2 and ~5 kDa, Figure 36) of the DGT-28 EPSPS protein. The DGT-28 EPSPS protein was incubated for 2 minutes in SGF and then incubated for 0, 0.5, 1, 2, 5, 10, 20, and 30 minutes in SIF.

Sequential pepsin and pancreatin digestion results indicated the low molecular weight bands (~2 and ~5 kDa) observed in SGF digestion (Figure 36) were digested within 0.5 minutes during sequential SIF digestion (Figure 40).

Additional details regarding analytical methods are provided in Appendix F.



Lane	Sample Descriptions
1	Pre-stained Protein Molecular Weight Marker[a]
2	DGT-28 EPSPS Protein in SGF, Time 0
3	SGF Only; 2 minutes
4	DGT-28 EPSPS Protein in SGF, 2 minutes
5	DGT-28 EPSPS Protein in SGF 2 minutes, SIF Time 0
6	DGT-28 EPSPS Protein in SGF 2 minutes, SIF 0.5 minutes
7	DGT-28 EPSPS Protein in SGF 2 minutes, SIF 1 minute
8	DGT-28 EPSPS Protein in SGF 2 minutes, SIF 2 minutes
9	DGT-28 EPSPS Protein in SGF 2 minutes, SIF 5 minutes
10	DGT-28 EPSPS Protein in SGF 2 minutes, SIF 10 minutes
11	DGT-28 EPSPS Protein in SGF 2 minutes, SIF 20 minutes
12	DGT-28 EPSPS Protein in SGF 2 minutes, SIF 30 minutes

Note: kilodalton (kDa), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF).

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

Figure 40. SDS-PAGE Analysis of DGT-28 EPSPS Protein in a Sequential Digestion with Simulated Gastric Fluid and Simulated Intestinal Fluid

Glycoprotein Analysis (PHI-2021-151 study)

As stated previously in in the characterization section, the results from glycoprotein staining analysis confirmed the absence of glycosylation for the DGT-28 EPSPS protein derived from DAS1131 maize tissue.

Evaluation of the Acute Toxicity of DGT-28 EPSPS Protein (PHI-2021-170 study)

A study was conducted to evaluate the potential acute toxicity of the test substance, DGT-28 EPSPS protein, in groups of 5 male and 5 female [redacted] mice following oral exposure at the limit dose (2000 mg/kg body weight, adjusted for DGT-28 EPSPS protein content). DGT-28 EPSPS protein was reconstituted in deionized water. Vehicle control and DGT-28 EPSPS protein formulations were administered orally by gavage. The mice were fasted prior to and throughout the dosing procedure.

Body weights were evaluated on test days 1 (prior to fasting and shortly prior to dose administration), 2, 3, 5, 8, and 15. Clinical signs were evaluated four times on test day 1 (before and after dosing) and daily thereafter. On test day 15, all mice were euthanized and given a gross pathological examination.

All animals survived to scheduled euthanasia. There were no clinical abnormalities and all animals gained weight during the 2-week observation period prior to euthanasia. No gross lesions were observed in this study.

Under the conditions of this study, intragastric exposure of the DGT-28 EPSPS protein to male and female mice at 2000 mg/kg body weight did not result in mortality or other evidence of acute oral toxicity, based on evaluation of body weight, clinical signs, and gross pathology. Therefore, the acute oral toxicity tolerant dose and the LD_{50} of DGT-28 EPSPS protein was determined to be greater than 2000 mg/kg body weight.

Conclusions on the Safety of DGT-28 EPSPS Protein in DAS1131 Maize

In conclusion, protein characterization results via SDS-PAGE, western blot, glycosylation, mass spectrometry, and N-terminal amino acid sequence analysis have demonstrated that the DGT-28 EPSPS protein derived from DAS1131 maize is of the expected molecular weight, immunoreactivity, lack of glycosylation, and amino acid sequence. Characterization of the microbially derived DGT-28 EPSPS protein demonstrated that it is an appropriate test substance for use in safety studies.

The allergenic and toxic potential of the DGT-28 EPSPS protein was assessed using a bioinformatic comparison of the amino acid sequence of the DGT-28 EPSPS protein to known or putative protein allergen and toxin sequences, evaluation of the stability of the DGT-28 EPSPS protein using *in vitro* gastric and intestinal digestion models, determination of the DGT-28 EPSPS protein glycosylation status, evaluation of the heat lability of the DGT-28 EPSPS protein using a enzymatic assay, and an evaluation of acute toxicity in mice following oral exposure to the DGT-28 EPSPS protein.

The bioinformatic comparisons of the DGT-28 EPSPS protein sequence to known and putative allergen and toxin sequences showed that the DGT-28 EPSPS protein is unlikely to be allergenic

or toxic to humans or animals. The DGT-28 EPSPS protein migrating at ~45 kDa was digested in SGF within 0.5 minutes, and some low molecular weight bands remained visible for up to 60 minutes. The protein was digested in SIF within 0.5 minutes. The low molecular weight bands remaining from SGF digestion were digested within 0.5 minutes in sequential SIF. The DGT-28 EPSPS protein was not glycosylated. The DGT-28 EPSPS protein heated for approximately 30-35 minutes at targeted temperatures of 50 °C or higher was inactive as demonstrated by enzymatic assay. The mouse acute oral toxicity assessment where no treatment-related effects were observed determined the LD_{50} of the DGT-28 EPSPS protein to be greater than the limit dose of 2000 mg/kg. These data support the conclusion that DAS1131 maize is as safe as conventional maize for the food and feed supply.

Based on this weight of evidence, consumption of the DGT-28 EPSPS protein from DAS1131 maize is unlikely to cause an adverse effect on humans or animals.

B.3 Other (non-protein substances)

There are no other new substances associated with DAS1131 maize.

B.4 Novel herbicide metabolites in GM herbicide-tolerant plants

There are no novel herbicide metabolites associated with DAS1131 maize. The mechanism of action or enzymatic reaction catalyzed by DGT-28 EPSPS is the same as other natural and characterized modified EPSP synthases that have been shown to confer tolerance to glyphosate, and there is a history of safe use in commercialized crops (CERA - ILSI Research Foundation, 2011).

B.5 Compositional analyses of the food produced using gene technology

Trait Expression Assessment (PHI-2020-019 study)

The expression levels of the Cry1Da2 and DGT-28 EPSPS proteins were evaluated in DAS1131 maize.

Tissue samples were collected during the 2020 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 (V6, V9, R1, and R4 growth stages), root (V9, R1, and R4 growth stages), pollen (R1 growth stage), stalk (R1 growth stage), forage (R4 growth stage), and grain (R6 growth stage). The concentrations of the Cry1Da2 and DGT-28 EPSPS proteins were determined using quantitative enzyme-linked immunosorbent assays (ELISAs).

Concentration results (means, ranges, and standard deviations) are summarized across sites in Table 30.

Table 31 for Cry1Da2 and DGT-28 EPSPS protein, respectively. Individual sample results below the LLOQ were assigned a value equal to half of the LLOQ for calculation purposes.

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

Table 30. Across-Sites Summary of Cry1Da2 Protein Concentrations in DAS1131 Maize

Tissue (Growth Stage)	ng Cry1Da2/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	**Range**	**Standard Deviation**	**Sample LLOQ[a]**	
			DAS1131 Maize		
Leaf (V6)	43	33 - 52	5.4	0.27	0/24
Leaf (V9)	31	19 - 52	7.6	0.27	0/24
Leaf (R1)	32	26 - 40	4.4	0.27	0/24
Leaf (R4)	37	26 - 60	6.9	0.27	0/24
Root (V9)	29	15 - 42	7.8	0.14	0/24
Root (R1)	19	13 - 30	4.2	0.14	0/24
Root (R4)	19	7.2 - 27	5.6	0.14	0/24
Pollen (R1)	46	36 - 62	8.2	0.54	0/24
Stalk (R1)	19	16 - 24	2.3	0.090	0/24
Forage (R4)	22	13 - 28	3.3	0.090	0/24
Grain (R6)	9.4	4.5 - 14	2.4	0.14	0/24

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

Table 31. Across-Sites Summary of DGT-28 EPSPS Protein Concentrations in DAS1131 Maize

Tissue (Growth Stage)	ng DGT-28 EPSPS/mg Tissue Dry Weight				Number of Samples <LLOQ/ Number of Samples Reported
	Mean	**Range**	**Standard Deviation**	**Sample LLOQ[a]**	
Leaf (V6)	44[b]	<22 - 78	17[c]	22	2/24[b]
Leaf (V9)	38[b]	<22 - 72	18[c]	22	3/24[b]
Leaf (R1)	66	39 - 130	26	22	0/24
Leaf (R4)	83	42 - 150	28	22	0/24
Root (V9)	12	7.2 - 21	3.9	5.4	0/24
Root (R1)	9.2[b]	<5.4 - 15	3.1[c]	5.4	2/24[b]
Root (R4)	4.1[b]	<5.4 - 8.7	2.2[c]	5.4	17/24[b]
Pollen (R1)	26[b]	<22 - 43	10[c]	22	6/24[b]
Stalk (R1)	21	12 - 38	6.7	3.6	0/24
Forage (R4)	38	16 - 74	13	7.2	0/24
Grain (R6)	28	16 - 48	7.8	5.4	0/24

Note: Growth stages (Abendroth *et al.*, 2011). Herbicide-treated refers to treatment with glyphosate.
[a] Lower limit of quantification (LLOQ) in ng/mg tissue dry weight.
[b] Some, but not all, sample results were below the LLOQ. A value equal to half the LLOQ value was assigned to those samples to calculate the mean and standard deviation.

Nutrient Composition Assessment (PHI-2020-021/021 study)

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 DAS1131 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 natural variation established from multiple sources of non-GM, commercial maize data.

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected during the 2020 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 DAS1131 maize, non-GM near-isoline control maize, and four non-GM commercial maize reference lines. A herbicide treatment of glyphosate was applied to DAS1131 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 DAS1131 maize and the control maize. Across-site comparisons were conducted for a total of 79 analytes, where 71 analytes were analyzed using mixed model analysis and 2 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 (Appendix H). For a given analyte in the mixed model analysis, if a statistical difference (P-value < 0.05) was observed between DAS1131 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 DAS1131 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 32. Nutrient composition analysis results are provided in Table 33 to Table 39. No statistically significant differences were observed between DAS1131 maize and the control maize for 66 of the 73 analytes that went through 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 DAS1131 maize and the control maize for seven analytes (moisture, palmitoleic acid [C16:1], manganese, zinc, calcium, β-carotene, and vitamin B5 [pantothenic acid]). After FDR adjustment, the FDR-adjusted P-values for moisture, palmitoleic acid [C16:1], zinc, calcium, and vitamin B5 [pantothenic acid] were not significant, indicating that the observed differences were likely false positives. All individual values for these analytes were within the tolerance interval, literature range, and/or in-study reference range, indicating DAS1131 maize is within the range of biological variation for these analytes and the statistical differences are not biologically meaningful.

While the raw and FDR-adjusted P-Values for manganese and β-carotene were significant, all individual values for manganese and β-carotene in DAS1131 maize were within the tolerance interval, literature range, and/or in-study reference range, indicating that DAS1131 maize is within the range of biological variation for these analytes and that the statistical difference is not biologically meaningful.

The results of the nutrient composition assessment demonstrate that nutrient composition of forage and grain derived from DAS1131 maize is 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 32. Outcome of Nutrient Composition Assessment Across Sites

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)							
Proximates, Fiber, and Mineral Composition	Crude Protein Crude Fat Crude Fiber ADF NDF Ash Carbohydrates Calcium Phosphorus	--	--	--	--	--	--
Grain (R6 Growth Stage)							
Proximates and Fiber Composition	Crude Protein Crude Fat Crude Fiber ADF NDF Total Dietary Fiber Ash Carbohydrates	Moisture (%)	--	--	--	--	--
Fatty Acid Composition	Palmitic Acid (C16:0) Heptadecanoic Acid (C17:0) 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) Behenic Acid (C22:0) Lignoceric Acid (C24:0)	Palmitoleic Acid (C16:1)	--	--	--	--	Lauric Acid (C12:0) Myristic Acid (C14:0) Heptadecenoic Acid (C17:1) Eicosadienoic Acid (C20:2)

Table 32. Outcome of Nutrient Composition Assessment Across Sites (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)							
Amino Acid Composition	Alanine Arginine Aspartic Acid Cystine Glutamic Acid Glycine Histidine Isoleucine Leucine Lysine Methionine Phenylalanine Proline Serine Threonine Tryptophan Tyrosine Valine	--	--	--	--	--	--
Mineral Composition	Copper Iron Magnesium Phosphorus Potassium Sodium	Manganese Zinc	Calcium	--	--	Manganese	--
Vitamin Composition	Vitamin B1 (Thiamine) Vitamin B2 (Riboflavin) Vitamin B3 (Niacin) Vitamin B6 (Pyridoxine) Vitamin B9 (Folic Acid) α-Tocopherol β-Tocopherol γ-Tocopherol Total Tocopherols	β-Carotene Vitamin B5 (Pantothenic Acid)	--	--	--	β-Carotene	δ-Tocopherol

Table 32. Outcome of Nutrient Composition Assessment Across Sites (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)							
Secondary Metabolite and Anti-Nutrient Composition	*p*-Coumaric Acid Ferulic Acid Inositol Phytic Acid Raffinose Trypsin Inhibitor	--	--	--	--	--	Furfural

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

Proximate, Fiber, and Mineral Assessment of DAS1131 Maize Forage

Proximates, fiber, and minerals were analyzed in forage derived from DAS1131 maize and control maize. Results are shown in Table 33. No statistically significant differences (P-value < 0.05) were observed between DAS1131 maize and control maize.

The results demonstrate that the proximate, fiber, and mineral composition of forage derived from DAS1131 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 33. Proximate, Fiber, and Mineral Results for DAS1131 Maize Forage

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Mean	7 72	7 73			
	Range	5 24 - 10 2	4 83 - 9 87			
Crude Protein	Confidence Interval	6 76 - 8 69	6 76 - 8 69	3 70 - 12 3	2 37 - 16 32	4 25 - 11 2
	Adjusted P-Value	--	0 962			
	P-Value	--	0 962			
	Mean	3 52	3 50			
	Range	2 05 - 4 76	2 49 - 4 88			
Crude Fat	Confidence Interval	3 20 - 3 84	3 18 - 3 82	0 867 - 6 50	NQ - 6 755	2 21 - 6 24
	Adjusted P-Value	--	0 906			
	P-Value	--	0 842			
	Mean	23 2	23 4			
	Range	18 2 - 31 7	15 7 - 30 8			
Crude Fiber	Confidence Interval	21 6 - 24 9	21 7 - 25 1	13 8 - 31 2	12 5 - 42	14 5 - 29 3
	Adjusted P-Value	--	0 906			
	P-Value	--	0 845			
	Mean	30 3	29 3			
	Range	23 6 - 38 7	21 5 - 34 0			
ADF	Confidence Interval	28 5 - 32 0	27 5 - 31 0	15 7 - 40 1	5 13 - 47 39	17 2 - 38 3
	Adjusted P-Value	--	0 637			
	P-Value	--	0 320			
	Mean	48 4	48 2			
	Range	39 6 - 61 0	42 0 - 54 2			
NDF	Confidence Interval	45 8 - 51 0	45 6 - 50 8	28 8 - 63 2	18 30 - 67 80	33 6 - 58 9
	Adjusted P-Value	--	0 934			
	P-Value	--	0 901			
	Mean	5 09	5 02			
	Range	3 39 - 8 46	2 74 - 6 50			
Ash	Confidence Interval	4 42 - 5 76	4 35 - 5 69	2 42 - 9 35	0 66 - 13 20	2 47 - 7 88
	Adjusted P-Value	--	0 886			
	P-Value	--	0 696			
	Mean	83 6	83 9			
	Range	78 0 - 87 3	80 7 - 87 0			
Carbohydrates	Confidence Interval	82 3 - 84 9	82 6 - 85 2	76 7 - 91 2	73 3 - 92 9	78 3 - 88 3
	Adjusted P-Value	--	0 637			
	P-Value	--	0 165			
	Mean	0 251	0 240			
	Range	0 123 - 0 472	0 0942 - 0 455			
Calcium	Confidence Interval	0 191 - 0 311	0 180 - 0 300	0 0768 - 0 528	0 04 - 0 58	0 0860 - 0 516
	Adjusted P-Value	--	0 726			
	P-Value	--	0 452			
	Mean	0 276	0 286			
	Range	0 202 - 0 344	0 231 - 0 397			
Phosphorus	Confidence Interval	0 249 - 0 302	0 260 - 0 313	0 0919 - 0 433	0 07 - 0 55	0 179 - 0 402
	Adjusted P-Value	--	0 637			
	P-Value	--	0 350			

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.

Proximate and Fiber Assessment of DAS1131 Maize Grain

Proximates and fiber were analyzed in grain derived from DAS1131 maize and control maize. Results are shown in Table 34. No statistically significant differences (P-value < 0.05) were observed between DAS1131 maize and control maize, with an exception for one analyte. A statistically significant difference (P-value < 0.05) was observed between DAS1131 maize and control maize for moisture. All individual values for this analyte were within the tolerance interval, indicating DAS1131 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 indicates that this difference was likely a false positive.

These results demonstrate that the proximate and fiber composition of grain derived from DAS1131 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 34. Proximate and Fiber Results for DAS1131 Maize Grain

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Mean	19 2	18 8			
	Range	7 41 - 29 3	7 11 - 28 7			
Moisture (%)	Confidence Interval	14 1 - 24 3	13 7 - 23 9	3 74 - 38 1	5 1 - 40 7	6 41 - 34 9
	Adjusted P-Value	--	0 377			
	P-Value	--	0 0212*			
	Mean	9 12	9 23			
	Range	6 88 - 10 9	7 27 - 10 3			
Crude Protein	Confidence Interval	8 37 - 9 88	8 48 - 9 98	6 60 - 13 1	5 72 - 17 26	6 14 - 11 8
	Adjusted P-Value	--	0 637			
	P-Value	--	0 283			
	Mean	3 54	3 39			
	Range	2 98 - 4 08	2 93 - 3 96			
Crude Fat	Confidence Interval	3 38 - 3 70	3 23 - 3 55	2 58 - 6 41	1 363 - 7 830	2 75 - 5 64
	Adjusted P-Value	--	0 611			
	P-Value	--	0 145			
	Mean	2 43	2 54			
	Range	1 95 - 3 19	1 91 - 3 32			
Crude Fiber	Confidence Interval	2 22 - 2 63	2 34 - 2 75	1 61 - 3 52	0 49 - 5 5	1 56 - 3 46
	Adjusted P-Value	--	0 611			
	P-Value	--	0 140			
	Mean	3 72	3 84			
	Range	2 82 - 5 31	2 71 - 4 97			
ADF	Confidence Interval	3 42 - 4 02	3 54 - 4 14	2 69 - 6 14	1 41 - 11 34	3 02 - 5 78
	Adjusted P-Value	--	0 637			
	P-Value	--	0 340			
	Mean	10 2	10 2			
	Range	8 91 - 12 2	8 51 - 13 2			
NDF	Confidence Interval	9 78 - 10 5	9 85 - 10 6	7 60 - 18 1	4 28 - 24 30	8 82 - 14 8
	Adjusted P-Value	--	0 895			
	P-Value	--	0 732			
	Mean	9 47	9 55			
	Range	8 15 - 11 4	7 80 - 11 5			
Total Dietary Fiber	Confidence Interval	9 06 - 9 88	9 14 - 9 96	2 97 - 20 0	5 78 - 35 31	7 35 - 13 0
	Adjusted P-Value	--	0 886			
	P-Value	--	0 712			
	Mean	1 25	1 25			
	Range	1 07 - 1 81	1 08 - 1 59			
Ash	Confidence Interval	1 13 - 1 37	1 13 - 1 37	1 01 - 1 83	0 616 - 6 282	1 01 - 1 76
	Adjusted P-Value	--	0 934			
	P-Value	--	0 921			
	Mean	86 1	86 1			
	Range	83 9 - 88 3	84 8 - 88 3			
Carbohydrates	Confidence Interval	85 3 - 86 9	85 3 - 86 9	80 6 - 88 7	77 4 - 89 7	82 3 - 89 2
	Adjusted P-Value	--	0 895			
	P-Value	--	0 755			

Fatty Acid Assessment of DAS1131 Maize Grain

Fatty acids were analyzed in grain derived from DAS1131 maize and control maize. Results are shown in Table 35. No statistically significant differences (P-value < 0.05) were observed between DAS1131 maize and control maize, with an exception for one analyte. A statistically significant difference (P-value < 0.05) was observed between DAS1131 maize and control maize for palmitoleic acid (C16:1). All individual values for this analyte were within the tolerance interval, indicating DAS1131 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 indicates that this difference was likely a false positive.

These results demonstrate that the fatty acid composition of grain derived from DAS1131 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 35. Fatty Acid Results for DAS1131 Maize Grain

Analyte	Reported Statistics	Control Maize	DAS1131 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	0 0302 - 0 0859
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Palmitic Acid (C16:0)	Mean	11 8	11 7			
	Range	11 3 - 12 7	11 3 - 12 6			
	Confidence Interval	11 5 - 12 1	11 4 - 12 0	9 51 - 24 5	6 81 - 39 0	10 8 - 15 4
	Adjusted P-Value	--	0 674			
	P-Value	--	0 399			
Palmitoleic Acid (C16:1)	Mean	0 134	0 130			
	Range	0 119 - 0 155	0 110 - 0 151			
	Confidence Interval	0 127 - 0 140	0 124 - 0 136	0 - 0 421	NQ - 0 67	0 0893 - 0 182
	Adjusted P-Value	--	0 406			
	P-Value	--	0 0452*			
Heptadecanoic Acid (C17:0)	Mean	0 0887	0 0907			
	Range	0 0462 - 0 112	0 0473 - 0 109			
	Confidence Interval	0 0740 - 0 103	0 0760 - 0 105	0 - 0 222	NQ - 0 203	0 0378 - 0 128
	Adjusted P-Value	--	0 637			
	P-Value	--	0 297			
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 82	1 84			
	Range	1 70 - 2 04	1 73 - 2 11			
	Confidence Interval	1 74 - 1 89	1 76 - 1 91	1 32 - 3 66	NQ - 4 9	1 35 - 2 92
	Adjusted P-Value	--	0 611			
	P-Value	--	0 133			
Oleic Acid (C18:1)	Mean	25 9	25 8			
	Range	24 3 - 28 2	24 5 - 28 5			
	Confidence Interval	25 3 - 26 5	25 2 - 26 4	17 2 - 38 0	16 38 - 42 81	20 8 - 42 5
	Adjusted P-Value	--	0 811			
	P-Value	--	0 537			
Linoleic Acid (C18:2)	Mean	56 9	57 2			
	Range	53 0 - 58 2	52 6 - 58 5			
	Confidence Interval	56 0 - 57 9	56 2 - 58 1	32 0 - 65 2	13 1 - 67 68	39 4 - 61 9
	Adjusted P-Value	--	0 637			
	P-Value	--	0 242			
α-Linolenic Acid (C18:3)	Mean	1 87	1 85			
	Range	1 64 - 2 21	1 59 - 2 20			
	Confidence Interval	1 78 - 1 97	1 75 - 1 95	0 - 2 09	NQ - 2 33	1 34 - 2 11
	Adjusted P-Value	--	0 726			
	P-Value	--	0 454			

Table 35. Fatty Acid Results for DAS1131 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
Arachidic Acid (C20:0)	Mean	0 422	0 421	0 297 - 0 811	0 267 - 1 2	0 305 - 0 650
	Range	0 379 - 0 537	0 370 - 0 522			
	Confidence Interval	0 388 - 0 455	0 387 - 0 454			
	Adjusted P-Value	--	0 868			
	P-Value	--	0 648			
Eicosenoic Acid (C20:1)	Mean	0 295	0 292	0 140 - 0 441	NQ - 1 952	0 228 - 0 390
	Range	0 260 - 0 348	0 262 - 0 353			
	Confidence Interval	0 279 - 0 312	0 275 - 0 309			
	Adjusted P-Value	--	0 637			
	P-Value	--	0 270			
Eicosadienoic Acid (C20:2)	Mean	<LLOQ[a]	<LLOQ[a]	0 - 0 825[r]	NQ - 2 551	0 0307 - 0 0817
	Range	<LLOQ[a]	<LLOQ[a]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Behenic Acid (C22:0)	Mean	0 227	0 219	0 - 0 424	NQ - 0 5	0 0763 - 0 368
	Range	0 0970 - 0 339	0 0992 - 0 328			
	Confidence Interval	0 186 - 0 268	0 178 - 0 259			
	Adjusted P-Value	--	0 406			
	P-Value	--	0 0596			
Lignoceric Acid (C24:0)	Mean	0 325	0 324	0 - 0 612	NQ - 0 91	0 211 - 0 470
	Range	0 282 - 0 442	0 270 - 0 453			
	Confidence Interval	0 291 - 0 359	0 290 - 0 358			
	Adjusted P-Value	--	0 934			
	P-Value	--	0 913			

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.

Amino Acid Assessment of DAS1131 Maize Grain

Amino acids were analyzed in grain derived from DAS1131 maize and control maize. Results are shown in Table 36. No statistically significant differences (P-value < 0.05) were observed between DAS1131 maize and control maize.

These results demonstrate that the amino acid composition of grain derived from DAS1131 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 36. Amino Acid Results for DAS1131 Maize Grain

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Mean	0 717	0 727			
	Range	0 492 - 0 874	0 538 - 0 868			
Alanine	Confidence Interval	0 647 - 0 787	0 657 - 0 797	0 448 - 1 06	0 40 - 1 48	0 457 - 0 960
	Adjusted P-Value	--	0 637			
	P-Value	--	0 296			
	Mean	0 402	0 406			
	Range	0 344 - 0 455	0 364 - 0 485			
Arginine	Confidence Interval	0 383 - 0 420	0 388 - 0 424	0 299 - 0 589	0 12 - 0 71	0 315 - 0 520
	Adjusted P-Value	--	0 637			
	P-Value	--	0 343			
	Mean	0 627	0 637			
	Range	0 443 - 0 776	0 484 - 0 763			
Aspartic Acid	Confidence Interval	0 571 - 0 683	0 581 - 0 693	0 412 - 0 890	0 30 - 1 21	0 414 - 0 815
	Adjusted P-Value	--	0 637			
	P-Value	--	0 266			
	Mean	0 195	0 205			
	Range	0 141 - 0 244	0 154 - 0 242			
Cystine	Confidence Interval	0 177 - 0 212	0 188 - 0 223	0 127 - 0 294	0 12 - 0 51	0 144 - 0 334
	Adjusted P-Value	--	0 406			
	P-Value	--	0 0522			
	Mean	1 81	1 85			
	Range	1 21 - 2 23	1 32 - 2 21			
Glutamic Acid	Confidence Interval	1 62 - 2 00	1 66 - 2 04	1 10 - 2 74	0 83 - 3 54	1 10 - 2 47
	Adjusted P-Value	--	0 637			
	P-Value	--	0 216			
	Mean	0 362	0 366			
	Range	0 313 - 0 402	0 322 - 0 414			
Glycine	Confidence Interval	0 345 - 0 379	0 349 - 0 382	0 285 - 0 481	0 184 - 0 685	0 302 - 0 453
	Adjusted P-Value	--	0 637			
	P-Value	--	0 279			

Table 36. Amino Acid Results for DAS1131 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Mean	0 286	0 288			
	Range	0 243 - 0 329	0 250 - 0 329			
Histidine	Confidence Interval	0 270 - 0 302	0 272 - 0 304	0 191 - 0 379	0 14 - 0 46	0 220 - 0 365
	Adjusted P-Value	--	0 812			
	P-Value	--	0 549			
	Mean	0 347	0 351			
	Range	0 256 - 0 413	0 283 - 0 417			
Isoleucine	Confidence Interval	0 318 - 0 376	0 322 - 0 380	0 207 - 0 491	0 18 - 0 69	0 233 - 0 448
	Adjusted P-Value	--	0 778			
	P-Value	--	0 504			
	Mean	1 17	1 19			
	Range	0 757 - 1 46	0 842 - 1 48			
Leucine	Confidence Interval	1 04 - 1 29	1 06 - 1 31	0 679 - 1 82	0 60 - 2 49	0 702 - 1 62
	Adjusted P-Value	--	0 637			
	P-Value	--	0 341			
	Mean	0 290	0 289			
	Range	0 249 - 0 342	0 254 - 0 330			
Lysine	Confidence Interval	0 275 - 0 304	0 274 - 0 303	0 178 - 0 397	0 129 - 0 668	0 223 - 0 367
	Adjusted P-Value	--	0 895			
	P-Value	--	0 765			
	Mean	0 209	0 215			
	Range	0 159 - 0 263	0 173 - 0 265			
Methionine	Confidence Interval	0 188 - 0 230	0 194 - 0 236	0 105 - 0 312	0 10 - 0 47	0 150 - 0 302
	Adjusted P-Value	--	0 637			
	P-Value	--	0 172			
	Mean	0 485	0 493			
	Range	0 361 - 0 577	0 397 - 0 576			
Phenylalanine	Confidence Interval	0 447 - 0 524	0 455 - 0 531	0 299 - 0 729	0 24 - 0 93	0 304 - 0 654
	Adjusted P-Value	--	0 637			
	P-Value	--	0 278			
	Mean	0 838	0 850			
	Range	0 595 - 0 981	0 651 - 0 979			
Proline	Confidence Interval	0 767 - 0 909	0 779 - 0 921	0 551 - 1 24	0 46 - 1 75	0 529 - 1 09
	Adjusted P-Value	--	0 637			
	P-Value	--	0 237			
	Mean	0 459	0 470			
	Range	0 348 - 0 541	0 386 - 0 541			
Serine	Confidence Interval	0 425 - 0 493	0 436 - 0 503	0 309 - 0 678	0 15 - 0 91	0 316 - 0 627
	Adjusted P-Value	--	0 506			
	P-Value	--	0 0855			
	Mean	0 350	0 359			
	Range	0 287 - 0 396	0 307 - 0 404			
Threonine	Confidence Interval	0 330 - 0 370	0 339 - 0 379	0 248 - 0 485	0 17 - 0 67	0 263 - 0 438
	Adjusted P-Value	--	0 406			
	P-Value	--	0 0628			
	Mean	0 0617	0 0619			
	Range	0 0507 - 0 0720	0 0511 - 0 0714			
Tryptophan	Confidence Interval	0 0579 - 0 0656	0 0581 - 0 0658	0 0373 - 0 0982	0 027 - 0 215	0 0480 - 0 0835
	Adjusted P-Value	--	0 906			
	P-Value	--	0 855			

Table 36. Amino Acid Results for DAS1131 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Mean	0 251	0 262			
	Range	0 164 - 0 349	0 176 - 0 343			
Tyrosine	Confidence Interval	0 227 - 0 274	0 239 - 0 286	0 149 - 0 510	0 10 - 0 73	0 147 - 0 354
	Adjusted P-Value	--	0 637			
	P-Value	--	0 240			
	Mean	0 451	0 455			
	Range	0 356 - 0 527	0 387 - 0 524			
Valine	Confidence Interval	0 421 - 0 482	0 425 - 0 485	0 300 - 0 627	0 21 - 0 86	0 328 - 0 549
	Adjusted P-Value	--	0 726			
	P-Value	--	0 460			

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

Mineral Assessment of DAS1131 Maize Grain

Minerals were analyzed in grain derived from DAS1131 maize and control maize. Results are shown in Table 37. No statistically significant differences (P-value < 0.05) were observed between DAS1131 maize and control maize, with a few exceptions. A statistically significant difference (P-value < 0.05) was observed between DAS1131 maize and control maize for manganese, zinc, and calcium. All individual values for these analytes were within the tolerance interval (manganese and zinc) or the literature range (calcium), indicating DAS1131 maize is within the range of biological variation for these analytes and the statistical difference is not biologically meaningful. The non-significant FDR-adjusted P-value (zinc and calcium) indicates that this difference was likely a false positive.

These results demonstrate that the mineral composition of grain derived from DAS1131 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 37. Mineral Results for DAS1131 Maize Grain

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
Calcium	Mean	0 00585	0 00532	0 00138 - 0 00735	NQ - 0 101	0 00245 - 0 00941
	Range	0 00327 - 0 0117	0 00350 - 0 00818			
	Confidence Interval	0 00484 - 0 00685	0 00431 - 0 00632			
	Adjusted P-Value	--	0 138			
	P-Value	--	0 00582[*]			
Copper	Mean	0 000132	0 000134	<0 0000625[b] - 0 000342	NQ - 0 0021	<0 0000625[b] - 0 000217
	Range	0 0000989 - 0 000228	0 0000942 - 0 000219			
	Confidence Interval	0 000104 - 0 000160	0 000106 - 0 000161			
	Adjusted P-Value	--	0 829			
	P-Value	--	0 584			
Iron	Mean	0 00164	0 00170	0 00115 - 0 00333	0 0000712 - 0 0191	0 00117 - 0 00279
	Range	0 00129 - 0 00200	0 00128 - 0 00211			
	Confidence Interval	0 00148 - 0 00179	0 00155 - 0 00186			
	Adjusted P-Value	--	0 611			
	P-Value	--	0 146			
Magnesium	Mean	0 111	0 112	0 0792 - 0 157	0 0035 - 1 000	0 0807 - 0 148
	Range	0 0895 - 0 131	0 0935 - 0 138			
	Confidence Interval	0 105 - 0 118	0 105 - 0 118			
	Adjusted P-Value	--	0 895			
	P-Value	--	0 769			
Manganese	Mean	0 000606	0 000654	0 000325 - 0 00120	0 0000312 - 0 0054	0 000389 - 0 000898
	Range	0 000472 - 0 00113	0 000502 - 0 00102			
	Confidence Interval	0 000514 - 0 000699	0 000561 - 0 000746			
	Adjusted P-Value	--	0 00676[†]			
	P-Value	--	<0 0001[*]			
Phosphorus	Mean	0 329	0 330	0 211 - 0 414	0 010 - 0 750	0 257 - 0 441
	Range	0 291 - 0 433	0 285 - 0 433			
	Confidence Interval	0 307 - 0 352	0 307 - 0 353			
	Adjusted P-Value	--	0 906			
	P-Value	--	0 825			
Potassium	Mean	0 333	0 336	0 238 - 0 504	0 020 - 0 720	0 266 - 0 521
	Range	0 296 - 0 453	0 295 - 0 452			
	Confidence Interval	0 303 - 0 362	0 306 - 0 366			
	Adjusted P-Value	--	0 650			
	P-Value	--	0 367			
Sodium	Mean	0 000383	0 000456	<LLOQ[b] - 0 0136	NQ - 0 15	<0 0000625[b] - 0 00958
	Range	<0 0000625[b] - 0 00985	<0 0000625[b] - 0 00599			
	Confidence Interval	0 000160 - 0 000917	0 000190 - 0 00109			
	Adjusted P-Value	--	0 868			
	P-Value	--	0 644			
Zinc	Mean	0 00195	0 00202	0 00133 - 0 00343	0 0000283 - 0 0043	0 00144 - 0 00334
	Range	0 00159 - 0 00238	0 00155 - 0 00265			
	Confidence Interval	0 00177 - 0 00213	0 00184 - 0 00219			
	Adjusted P-Value	--	0 406			
	P-Value	--	0 0454[*]			

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.

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

Vitamin Assessment of DAS1131 Maize Grain

Vitamins were analyzed in grain derived from DAS1131 maize and control maize. Results are shown in Table 38. No statistically significant differences (P-value < 0.05) were observed between DAS1131 maize and control maize, with a few exceptions. A statistically significant difference (P-value < 0.05) was observed between DAS1131 maize and control maize for β-carotene and vitamin B5 (pantothenic acid). All individual values for this analyte were within the tolerance interval, indicating DAS1131 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 (vitamin B5) indicates that this difference was likely a false positive.

These results demonstrate that the vitamin composition of grain derived from DAS1131 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 38. Vitamin Results for DAS1131 Maize Grain

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
	Mean	0 190	0 146			
	Range	<0 0500[b] - 0 353	<0 0500[b] - 0 300			
β-Carotene	Confidence Interval	0 114 - 0 265	0 0703 - 0 222	0 - 3 86	0 3 - 5 81	<0 0500[b] - 0 940
	Adjusted P-Value	--	0 0396[†]			
	P-Value	--	0 00112[*]			
	Mean	1 88	1 83			
	Range	<0 900[b] - 2 77	1 28 - 2 61			
Vitamin B1 (Thiamine)	Confidence Interval	1 72 - 2 05	1 66 - 1 99	1 12 - 4 85	NQ - 40 00	<0 900[b] - 4 71
	Adjusted P-Value	--	0 611			
	P-Value	--	0 129			
	Mean	<0 900[b]	0 473			
	Range	<0 900[b]	<0 900[b] - 1 18			
Vitamin B2 (Riboflavin)	Confidence Interval	NA	NA	<0 900[b] - 2 27[r]	NQ - 7 35	<0 900[b] - 1 52
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
	Mean	12 5	12 8			
	Range	10 1 - 19 9	10 2 - 15 2			
Vitamin B3 (Niacin)	Confidence Interval	11 7 - 13 4	11 9 - 13 6	7 75 - 30 5	NQ - 70	9 96 - 20 8
	Adjusted P-Value	--	0 829			
	P-Value	--	0 572			
	Mean	4 61	4 45			
	Range	3 32 - 6 17	3 33 - 5 51			
Vitamin B5 (Pantothenic Acid)	Confidence Interval	4 24 - 4 99	4 08 - 4 82	2 54 - 7 64	3 01 - 14	3 27 - 7 35
	Adjusted P-Value	--	0 406			
	P-Value	--	0 0339[*]			
	Mean	4 76	4 82			
	Range	2 55 - 7 08	2 99 - 7 07			
Vitamin B6 (Pyridoxine)	Confidence Interval	3 95 - 5 58	4 00 - 5 64	0 909 - 9 00	NQ - 12 14	2 67 - 7 95
	Adjusted P-Value	--	0 906			
	P-Value	--	0 855			
	Mean	3 58	4 22			
	Range	1 38 - 6 92	1 87 - 7 79			
Vitamin B9 (Folic Acid)	Confidence Interval	2 79 - 4 38	3 43 - 5 02	0 246 - 4 29	NQ - 3 50	1 26 - 6 86
	Adjusted P-Value	--	0 406			
	P-Value	--	0 0509			
	Mean	13 4	13 5			
	Range	4 59 - 21 4	4 45 - 20 4			
α-Tocopherol	Confidence Interval	9 77 - 17 0	9 91 - 17 2	0 - 22 7	NQ - 68 67	1 85 - 22 5
	Adjusted P-Value	--	0 852			
	P-Value	--	0 612			
	Mean	0 344	0 333			
	Range	<0 500[b] - 0 759	<0 500[b] - 0 759			
β-Tocopherol	Confidence Interval	NA	NA	<0 500[b] - 1 10[r]	NQ - 19 80	<0 500[b] - 1 05
	Adjusted P-Value	--	NA			
	P-Value	--	NA			

Table 38. Vitamin Results for DAS1131 Maize Grain (continued)

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
γ-Tocopherol	Mean	6 98	6 90	0 - 44 4	NQ - 58 61	3 15 - 38 1
	Range	4 03 - 19 7	4 22 - 11 0			
	Confidence Interval	5 50 - 8 46	5 42 - 8 38			
	Adjusted P-Value	--	0 886			
	P-Value	--	0 701			
δ-Tocopherol	Mean	<0 500[b]	<0 500[b]	<0 500[b] - 2 61[r]	NQ - 14 61	<0 500[b] - 1 81
	Range	<0 500[b]	<0 500[b]			
	Confidence Interval	NA	NA			
	Adjusted P-Value	--	NA			
	P-Value	--	NA			
Total Tocopherols	Mean	21 1	20 9	0 - 57 9	NQ - 89 91	8 58 - 53 5
	Range	9 94 - 29 3	9 17 - 28 8			
	Confidence Interval	16 8 - 25 3	16 7 - 25 2			
	Adjusted P-Value	--	0 880			
	P-Value	--	0 669			

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.

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

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

Secondary Metabolitie and Anti-Nutrient Assessment of DAS1131 Maize Grain

Secondary metabolite and anti-nutrients were analyzed in grain derived from DAS1131 maize and control maize. Results are shown in Table 39. No statistically significant differences (P-value < 0.05) were observed between DAS1131 maize and control maize.

These results demonstrate that the secondary metabolite and anti-nutrient composition of grain derived from DAS1131 maize is comparable to conventional maize represented by non-GM near-isoline control maize and non-GM commercial maize.

Table 39. Secondary Metabolite and Anti-Nutrient Results for DAS1131 Maize Grain

Analyte	Reported Statistics	Control Maize	DAS1131 Maize	Tolerance Interval	Literature Range	Reference Data Range
p-Coumaric Acid	Mean	0 0269	0 0278	0 00781 - 0 0483	NQ - 0 08	0 0131 - 0 0428
	Range	0 0175 - 0 0347	0 0168 - 0 0350			
	Confidence Interval	0 0238 - 0 0299	0 0248 - 0 0309			
	Adjusted P-Value	--	0 637			
	P-Value	--	0 182			
Ferulic Acid	Mean	0 273	0 280	0 131 - 0 343	0 02 - 0 44	0 140 - 0 308
	Range	0 216 - 0 341	0 199 - 0 316			
	Confidence Interval	0 261 - 0 285	0 267 - 0 292			
	Adjusted P-Value	--	0 637			
	P-Value	--	0 270			
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 0226	0 0221	0 00949 - 0 0571	0 00613 - 0 257	0 0125 - 0 0625
	Range	0 0124 - 0 0396	0 0134 - 0 0451			
	Confidence Interval	0 0160 - 0 0291	0 0156 - 0 0286			
	Adjusted P-Value	--	0 637			
	P-Value	--	0 349			
Phytic Acid	Mean	0 803	0 824	0 505 - 1 32	NQ - 1 940	0 616 - 1 13
	Range	0 602 - 1 12	0 627 - 1 00			
	Confidence Interval	0 727 - 0 880	0 748 - 0 900			
	Adjusted P-Value	--	0 637			
	P-Value	--	0 309			
Raffinose	Mean	0 108	0 104	0 - 0 387	NQ - 0 466	<0 0800[b] - 0 509
	Range	<0 0800[b] - 0 329	<0 0800[b] - 0 284			
	Confidence Interval	0 0726 - 0 144	0 0686 - 0 140			
	Adjusted P-Value	--	0 906			
	P-Value	--	0 795			
Trypsin Inhibitor (TIU/mg DW)	Mean	2 35	2 30	1 06 - 8 12	NQ - 8 42	1 45 - 3 78
	Range	1 36 - 3 57	1 45 - 3 34			
	Confidence Interval	2 07 - 2 64	2 01 - 2 58			
	Adjusted P-Value	--	0 650			
	P-Value	--	0 375			

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.

C. INFORMATION RELATED TO THE NUTRITIONAL IMPACT OF THE FOOD

In Section *B.5 Compositional analyses of the food produced using gene technology*, the compositional equivalence of DAS1131 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 DAS1131 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 DAS1131 maize are comparable to conventional maize with respect to nutrient composition.

Therefore, no nutritional impact of DAS1131 is expected.

D. OTHER INFORMATION

Overall Risk Assessment Conclusions for DAS1131 Maize

This application presents information supporting the safety and nutritional comparability of DAS1131 maize. The molecular characterization analyses conducted on DAS1131 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 Cry1Da2 and DGT-28 EPSPS proteins were evaluated and found unlikely to be allergenic or toxic to humans. Based on the weight of evidence, consumption of the Cry1Da2 and DGT-28 EPSPS proteins is unlikely to cause an adverse effect to humans. A compositional equivalence assessment demonstrated that the nutrient composition of DAS1131 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 DAS1131 maize containing the Cry1Da2 and DGT-28 EPSPS proteins is as safe and nutritious as non-GM maize.

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(2021) "Sequence Characterization of Insert and Flanking Genomic Regions of DAS-Ø1131-3 Maize" Corteva Agriscience study ID: PHI-2020-173

(2021) "Expressed Trait Protein Concentration of a Maize Line Containing Event DAS-Ø1131-3" Corteva Agriscience study ID: PHI-2020-019

et al. (2021) "Nutrient Composition of an Herbicide-Treated Maize Line Containing Event DAS-Ø1131-3" Corteva Agriscience study ID: PHI-2020-021/021

(2021) "Development and Validation of an Event-Specific Quantitative Real-Time PCR (qPCR) Detection Method for Maize Event DAS-Ø1131-3" Corteva Agriscience study ID: PHI-2021-042

(2021) "Characterization of the Genomic Border Regions of Maize Event DAS-Ø1131-3" Corteva Agriscience study ID: PHI-2021-171/230

█ (2021) "Comparison of the Amino Acid Sequence of the Cry1Da2 Protein to the Amino Acid Sequences of Known and Putative Protein Allergens" Corteva Agriscience study ID: PHI-2021-073/201

█ (2021) "Comparison of the Amino Acid Sequence of the DGT-28 EPSPS Protein to the Amino Acid Sequences of Known and Putative Protein Allergens" Corteva Agriscience study ID: PHI-2021-074/201

█ (2021) "Comparison of the Cry1Da2 Protein Sequence to the Protein Sequences in the Internal Toxin Database" Corteva Agriscience study ID: PHI-2021-208/211

█ (2021) "Comparison of the DGT-28 EPSPS Protein Sequence to the Protein Sequences in the Internal Toxin Database" Corteva Agriscience study ID: PHI-2021-209/211

█ (2021) "Reading Frame Analysis at the Insertion Site of Maize Event DAS-Ø1131-3" Corteva Agriscience study ID: PHI-2021-173/225

█ et al. (2022) "Characterization of Cry1Da2 Protein Derived from DAS-Ø1131-3 Maize" Corteva Agriscience study ID: PHI-2021-148

█ (2021) "Characterization of DGT-28 EPSPS Protein Derived from DAS-Ø1131-3 Maize" Corteva Agriscience study ID: PHI-2021-151

APPENDIX A. METHODS FOR SOUTHERN-BY-SEQUENCING ANALYSIS

Test and Control Substances

The test substance in this study was defined as the DAS-Ø1131-3 event contained within seed from the segregating T1 generation of DAS1131 maize. The control substance was defined as the absence of the DAS-Ø1131-3 event in untransformed B104 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 DAS1131 insertion.

DNA Extraction and Quantitation

Genomic DNA was separately extracted from leaf tissue of ten individual DAS1131 maize plants and one control maize plant. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep), and the genomic DNA was isolated using a standard Urea Extraction Buffer procedure. Following extraction, the DNA was quantified on a spectrofluorometer using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) and visualized on an agarose gel to determine the DNA quality.

Southern-by-Sequencing

SbS was performed by Pioneer Hi-Bred International, Inc. Genomics Technologies (hereafter referred to as 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 linearized transformation plasmid map and control maize genome, 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 sequences.

Genomic DNA isolated from the T1 generation of DAS1131 maize was analyzed by SbS to determine the insertion copy number and organization. SbS was also performed on control maize DNA and a positive control sample (control maize DNA spiked with PHP88492 plasmid DNA at a level corresponding to one copy of plasmid per copy of the maize genome) to confirm that the assay could reliably detect plasmid fragments within the genomic DNA.

The following processes were performed by 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 within the PHP88492 plasmid.

Sequencing Library Construction

NGS libraries were constructed for DNA samples from individual maize plants, including DAS1131 maize plants, a control maize plant, and the positive control sample. Genomic DNA purified as described above was sheared to an average fragment size of 400 base pairs (bp) using an ultrasonicator. Sheared DNA was end-repaired, A-tailed, and ligated to NEXTflex-HT™ Barcode adaptors (Bioo Scientific Corp.) following the kit protocol so that samples would be indexed to enable identification after sequencing. The DNA fragment libraries were amplified by PCR for eight cycles prior to the capture process. Amplified libraries were analyzed using a fragment analyzer and diluted to 5 ng/µl with nuclease-free water.

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 NextSeq500) to a depth of at least 100x for the captured sequences. The sequence reads were trimmed for quality below Q20 (Ewing and Green, 1998; Ewing *et al.*, 1998) and assigned to the corresponding individual plant based on the indexing adapters. A complete sequence set from each plant is referred to as “AllReads” for bioinformatics analysis of that plant.

Quality Assurance of Sequncing Reads

The adapter sequences were trimmed from the NGS sequence using Cutadapt, v2.10 (Martin, 2011). Further analysis to eliminate sequencing errors used JELLYFISH, version 2.2.10 (Marçais and Kingsford, 2011), to exclude any 31-bp sequence that occurred less than twice within “AllReads” as described in Zastrow-Hayes et al. (2015). This set of sequences was used for further bioinformatics analysis and is referred to as “CleanReads.” Identical sequence reads were combined into non-redundant read groups (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), with one mismatch allowed. Remaining "Non-redundantReads" were aligned to the maize reference genome using Bowtie2, version 2.3.4.2, with up to two mismatches allowed.

Junction Detection

Following removal of "Non-redundantReads" with alignments to the maize reference genome or plasmid sequence identified during the quality assurance phase, the remaining "Non-redundantReads" were aligned to the full plasmid sequence 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. The individual reads defining a junction were condensed to a unique identifier to represent the junction. This identifier (referred to as a 30_20 mer) includes 20 bp of sequence from PHP88492 and 30 bp of sequence adjacent to the plasmid-derived 20 bp within a sequencing read. The adjacent 30 bp either did not align to the plasmid contiguously to the known 20 bp or aligned to the genome. 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. Junction reads were condensed into a unique junction if their 30_20 mers were identical, or if the 30_20 mer junctions were within 2 bp. The total number of sequence reads (referred to as "TotalSupportingReads") for each unique junction was retained for filtering. Junctions with fewer than 10 "TotalSupportingReads" for positions aligned to the plasmid were filtered and removed from further analysis.

Junction Identification

Variations between the maize reference genome used in the SbS analysis and the control maize genome may result in identification of junctions that are due to these differences in the endogenous maize sequences. In order to detect these endogenous junctions, control maize genomic DNA libraries were captured and sequenced in the same manner. The 30_20 mers of the endogenous junctions detected in the control sample were used to filter the same endogenous junctions in the DAS1131 maize samples, so that the only junctions remaining in the DAS1131 samples are due to actual insertions derived from PHP88492.

Data QC

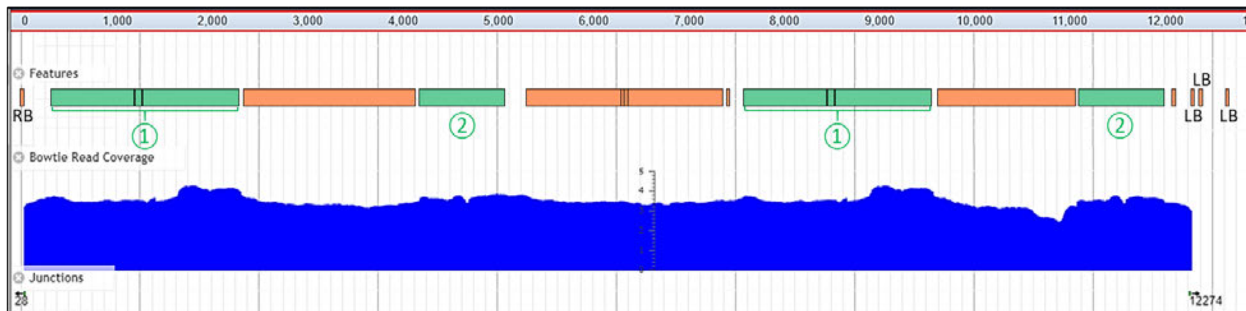
The transgenic and null samples were compared to the control sample and a quality check was performed. If a junction was identified between two noncontiguous plasmid regions and after further review was identified to be a PCR amplicon, then it was masked in the final data. PCR amplicons are usually found in regions that have a duplicated sequence (within the plasmid or adapter) similar enough that a sequencing read can align to either sequence and function as a primer to initiate spurious PCR amplicons.

If regions of the plasmid backbone contain low to medium sequencing coverage compared to the control sample and no junctions were identified, the data was reviewed a second time. If no junctions were identified for these reads, there is no insertion of the plasmid 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. If a null sample has additional regions on the map of the insertion that have low sequencing coverage and no junctions are identified, this is contamination from a transgenic plant.

SbS Results

Results for the control maize, positive control, one DAS1131 maize plant (Plant ID 404421230), and null segregant (negative) plant (Plant ID 404421227) are presented in the main body (Section 4a) of this document. Remaining plant results from SbS analysis are presented in Figures A1 to A5 (positive plants) below:

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

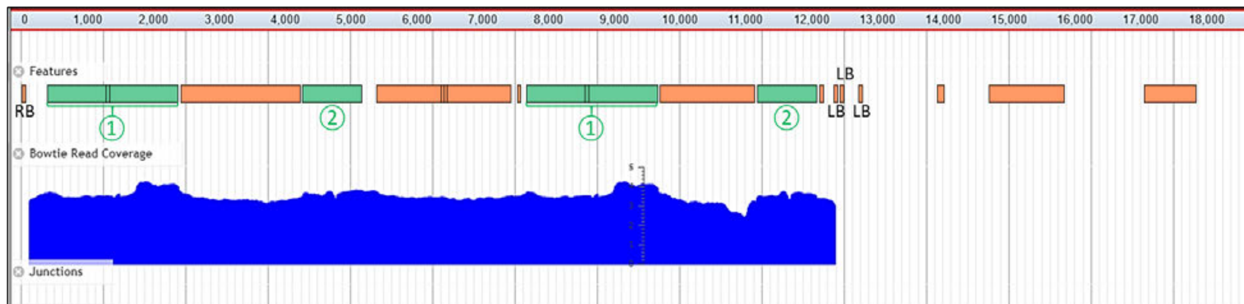
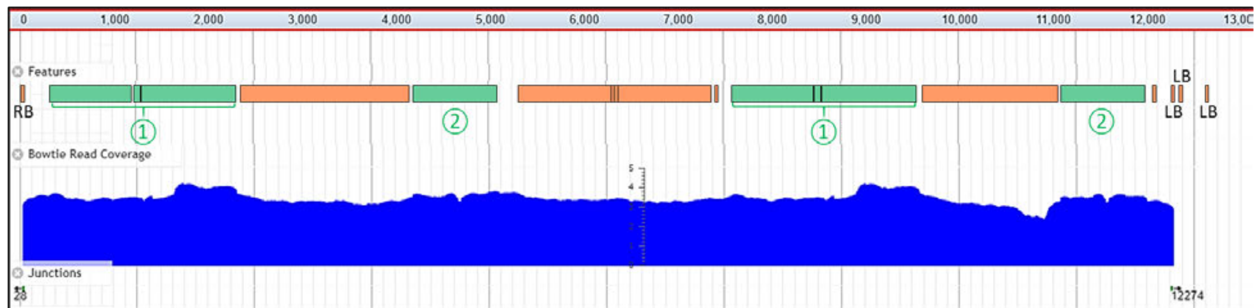


Figure A1. SbS Results for Plant ID 404421222 – DAS1131 Maize (Transgenic)

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 3), while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2) indicating that this plant contains the insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers below the arrows refer to the bp location of the junction relative to the PHP88492 T-DNA (Figure 2). The insertion comprises bp 28 to 12,274 of the PHP88492 T-DNA shown in Figure 2. The presence of only two junctions demonstrates the presence of a single insertion in the DAS1131 maize genome. **B)** SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 1). Coverage was obtained for the elements between the Right and Left Borders transferred into DAS1131 maize (region between the RB and LB elements); however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP88492 sequence shows that there are no additional insertions or backbone sequence present in DAS1131 maize.

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

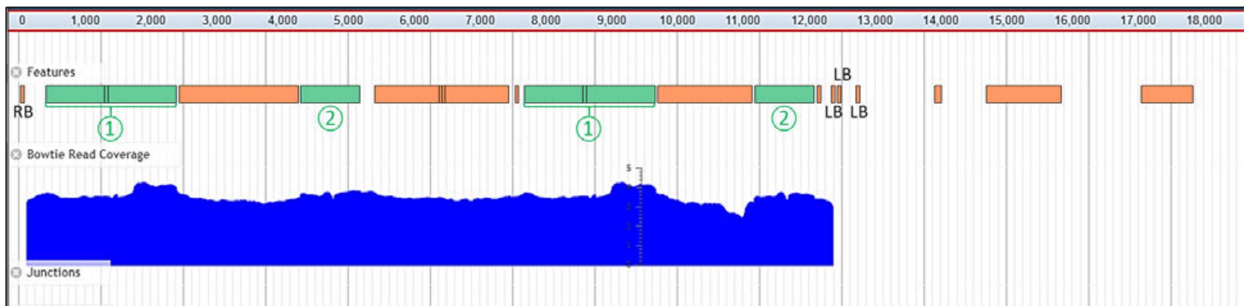
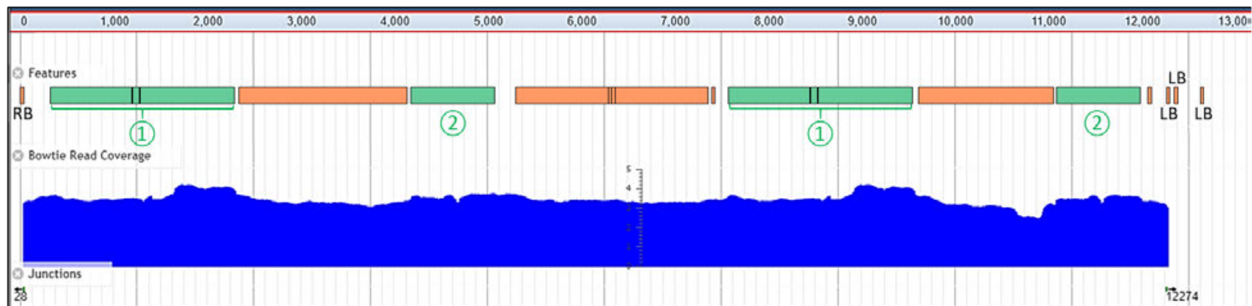


Figure A2. SbS Results for Plant ID 404421223 – DAS1131 Maize (Transgenic)

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 3), while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2) indicating that this plant contains the insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers below the arrows refer to the bp location of the junction relative to the PHP88492 T-DNA (Figure 2). The insertion comprises bp 28 to 12,274 of the PHP88492 T-DNA shown in Figure 2. The presence of only two junctions demonstrates the presence of a single insertion in the DAS1131 maize genome. **B)** SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 1). Coverage was obtained for the elements between the Right and Left Borders transferred into DAS1131 maize (region between the RB and LB elements); however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP88492 sequence shows that there are no additional insertions or backbone sequence present in DAS1131 maize.

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

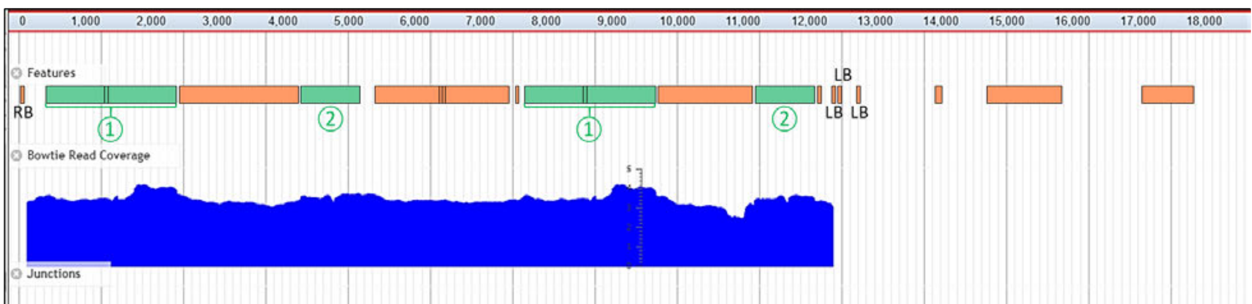
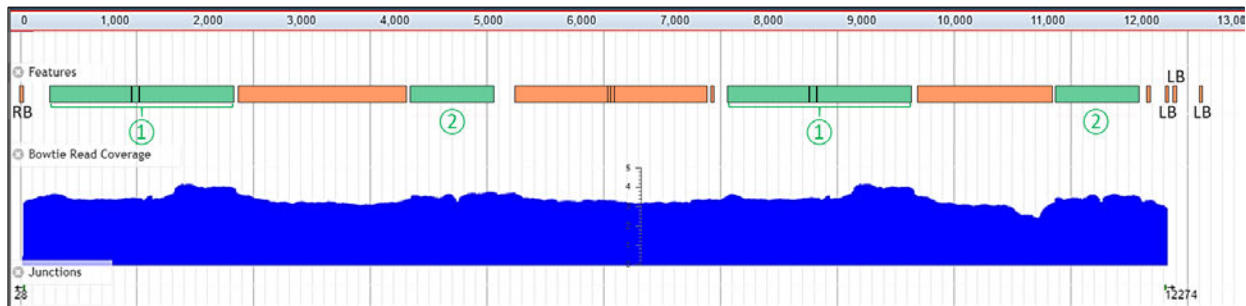


Figure A3. SbS Results for Plant ID 404421224 – DAS1131 Maize (Transgenic)

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 3), while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2) indicating that this plant contains the insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers below the arrows refer to the bp location of the junction relative to the PHP88492 T-DNA (Figure 2). The insertion comprises bp 28 to 12,274 of the PHP88492 T-DNA shown in Figure 2. The presence of only two junctions demonstrates the presence of a single insertion in the DAS1131 maize genome. **B)** SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 1). Coverage was obtained for the elements between the Right and Left Borders transferred into DAS1131 maize (region between the RB and LB elements); however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP88492 sequence shows that there are no additional insertions or backbone sequence present in DAS1131 maize.

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

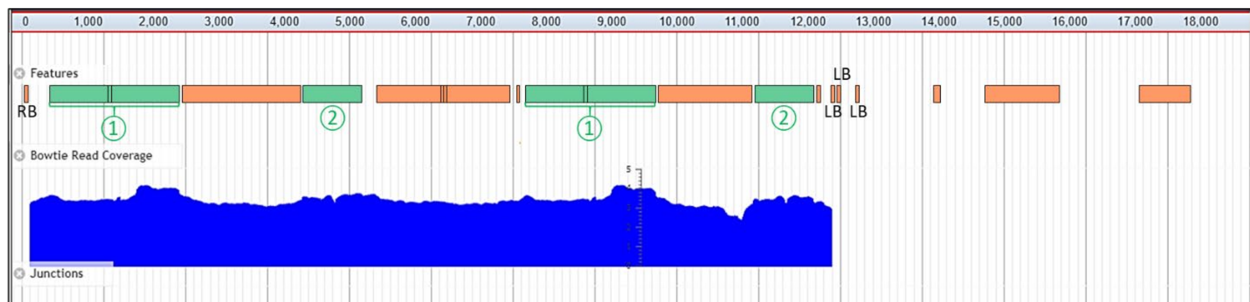
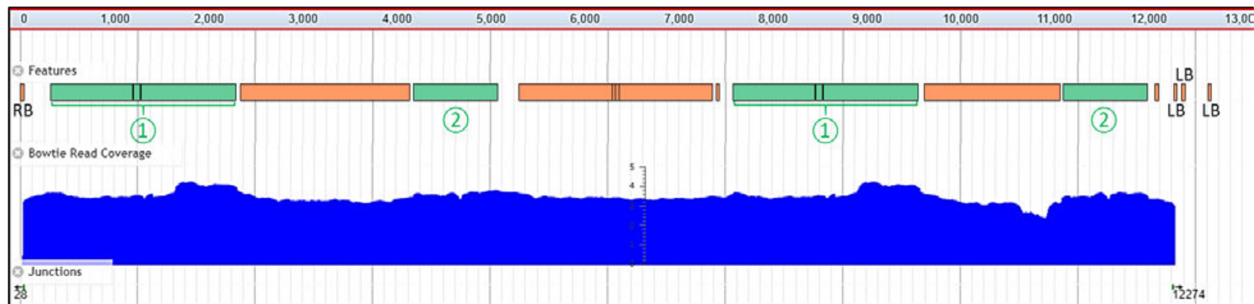


Figure A4. SbS Results for Plant ID 404421225 – DAS1131 Maize (Transgenic)

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 3), while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2) indicating that this plant contains the insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers below the arrows refer to the bp location of the junction relative to the PHP88492 T-DNA (Figure 2). The insertion comprises bp 28 to 12,274 of the PHP88492 T-DNA shown in Figure 2. The presence of only two junctions demonstrates the presence of a single insertion in the DAS1131 maize genome. **B)** SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 2). Coverage was obtained for the elements between the Right and Left Borders transferred into DAS1131 maize (region between the RB and LB elements); however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP88492 sequence shows that there are no additional insertions or backbone sequence present in DAS1131 maize.

A. Alignment to PHP88492 T-DNA Region



B. Alignment to PHP88492

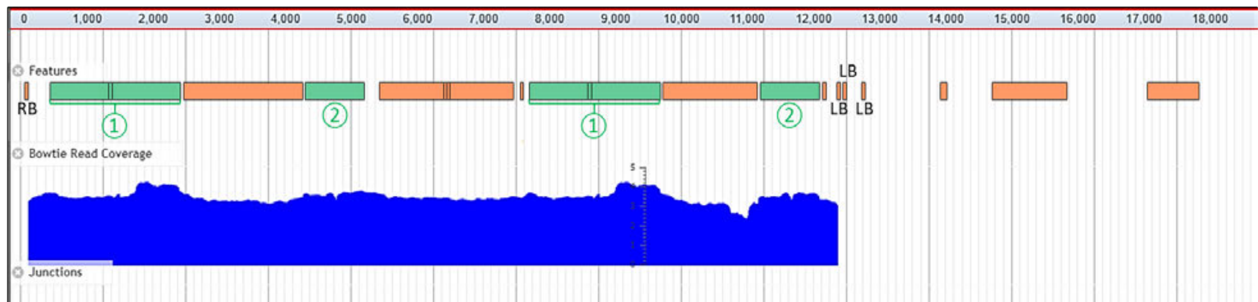


Figure A5. SbS Results for Plant ID 404421228 – DAS1131 Maize (Transgenic)

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 3), while tan bars indicate genetic elements derived from other sources. **A)** SbS results aligned against the PHP88492 T-DNA region intended for insertion (12,664 bp; Figure 2) indicating that this plant contains the insertion. Arrows below the graph indicate the two plasmid-to-genome sequence junctions identified by SbS; the numbers below the arrows refer to the bp location of the junction relative to the PHP88492 T-DNA (Figure 2). The insertion comprises bp 28 to 12,274 of the PHP88492 T-DNA shown in Figure 2. The presence of only two junctions demonstrates the presence of a single insertion in the DAS1131 maize genome. **B)** SbS results aligned against the plasmid PHP88492 sequence (18,024 bp; Figure 2). Coverage was obtained for the elements between the Right and Left Borders transferred into DAS1131 maize (region between the RB and LB elements); however, for clarity the junctions identified in panel A are not shown in this view. The absence of any other junctions to the PHP88492 sequence shows that there are no additional insertions or backbone sequence present in DAS1131 maize.

APPENDIX B. METHODS FOR SOUTHERN BLOT ANALYSIS

Test, Control and Reference Substances

The test substances in the study were defined as seeds from DAS1131 maize of the T1, T2, T3, T4, and T6 generations. The control substance was defined as seed from a maize line (B104) that was not transformed. B104 maize has a similar genetic background to the test substance; however, it does not contain the DAS1131 maize insertion.

Plasmid DNA of PHP88492 that was used for transformation to produce DAS1131 maize was defined as a reference substance. This plasmid was used as a positive control for Southern analysis to verify probe hybridization. The *cry*1Da2 and *dgt-28 epsps* probes used were derived from plasmid PHP88492.

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 DAS1131 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°C 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 leaf tissue of one plant each from five generations (T1, T2, T3, T4, and T6) of DAS1131 maize and one plant from the B104 control maize.

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. The extracted DNA was treated with Ribonuclease A (RNase A) and purified using phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated using sodium acetate and chilled ethanol. The purified 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 (10 µg) isolated from both test and control maize leaves was double digested with the restriction enzymes [redacted] and [redacted]). PHP88492 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 enzymes, the fragments produced were electrophoretically separated on a 0.9% agarose gel according to their sizes and 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 nylon membrane by UV crosslinking (UV Stratalinker, UVP).

Probe Labeling and Southern Blot Hybridization

DNA probes specific to the *cry*1Da2 and *dgt-28 epsps* gene elements (Figure 12) were labeled by incorporation of Digoxigenin (DIG) labeled nucleotide [DIG-11-dUTP] into the fragments by PCR labeling method.

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 with 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. Blots were exposed for one or more time points to detect hybridized fragments and to visualize molecular weight standards. Images were captured by 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 probes to prepare the blots 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 Saline sodium citrate 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

Five generations of DAS1131 maize (BC1F1 [B104/PH184C], BC1F1 [B104/PH1V5T], T2, T4, and T6 generations) were evaluated using real-time quantitative PCR (qPCR) and herbicide-tolerance testing to confirm Mendelian inheritance of genotype and phenotype.

Planting and Leaf Sample Collection

Approximately 160 seeds per generation were planted in separate pots and grown in a controlled environment under suitable environmental conditions for producing maize plants. After germination, each generation was thinned to a final population of 100 healthy plants prior to any sampling or analysis.

One leaf sample per plant was collected at the V2-V3 growth stage (occurs when the leaflets on the second or third leaf node, respectively, have unrolled). Each sample consisted of three leaf punches collected into one bullet tube and placed on dry ice until transferred to a freezer for frozen storage. Individual plants and corresponding leaf punch samples were uniquely labeled to allow a given sample to be tracked back to the originating plant.

Genotypic Analysis

DNA extraction was performed for the collected leaf samples. The extracted samples were analyzed using a qPCR assay to confirm the copy number of event DAS-Ø1131-3 and associated genes.

Phenotypic Analysis

A glyphosate herbicide treatment was applied after PCR leaf punch sample collection, when plants were at the V4 growth stage (occurs when the leaflets on the fourth leaf node have unrolled). The spray mixture consisted of Durango DMA containing 4 pounds of glyphosate acid equivalent per gallon (0.5 kg ae/L) and ammonium sulfate at a rate of approximately 3.0 pounds per acre. No other adjuvants or additives were included in the spray mixture. Durango DMA was applied at a target rate of 2.25 pints per acre (2.6 L/ha) with a total spray volume of approximately 33 gal/A (309 L/ha) using a spray chamber to simulate a broadcast (over-the-top) application. Actual application rates were within ±10% of the target herbicide application rate.

Seven 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.

Genotypic and phenotypic results for each plant were compared to verify that qPCR assay results correlated with herbicide tolerance results.

<u>Statistical Analysis</u>

A chi-square test was performed at the 0.05 significance level on the segregation results of BC1F1 (Entries 1 and 2) and T2 generations of DAS1131 maize. The observed segregation ratio was determined by combining genotype and phenotype results. All plants were identified as positive or negative with positive plants tolerant to glyphosate and confirmed to contain all the transgenes (one or two copy), and negative plants susceptible to glyphosate and not containing the transgenes (NULL). A chi-square test was performed separately for each generation to compare the observed segregation ratio to the expected segregation ratio (1:1 for BC1F1, 3:1 for T2). Statistical analyses were conducted using SAS software, Version 9.4.

APPENDIX D. METHODS FOR SANGER SEQUENCING ANALYSIS

Test and Control Substances

The test substance in the study was defined as seeds from DAS1131 maize of the T3 generation. The control substance was defined as seed from a non-genetically modified (non-GM) maize line, B104, that has a similar genetic background to the test substance but does not contain the DAS1131 maize insertion.

DNA Extraction and Quantification

Genomic DNA was extracted from pooled leaf tissue of equal amounts from 10 DAS1131 maize plants and a separate pool of leaf tissue from two control maize plants.

Genomic DNA was extracted from finely ground maize leaf tissue using a urea lysis buffer, purified using phenol/chloroform/isoamyl alcohol (25:24:1) separation, RNase treatment, DNA precipitation, and spooling. For DAS1131 maize, approximately 0.5 grams of leaf tissue was collected from each of 10 plants, pooled, and ground into a fine powder for DNA extraction. For control maize, approximately 0.5 grams of leaf tissue was collected from each of two plants, pooled, and ground into a fine powder for DNA extraction.

The presence of the DAS1131 insertion in the extracted genomic DNA from the DAS1131 maize plants and the absence in the extracted DNA from the control maize were further confirmed by event-specific quantitative real-time PCR.

Polymerase Chain Reaction (PCR) Amplification of the Insert and Flanking Genomic Regions in DAS1131 Maize

Eight overlapping PCR fragments (**A**, **B**, **C**, **D**, **E**, **F**, **G**, and **H**) spanning the insert and the 5' and 3' flanking border regions were amplified from the genomic DNA of DAS1131 maize. The non-GM control maize DNA was also used in amplification reactions under the same PCR conditions to serve as a negative control. Amplification conditions specific to the generation of each PCR fragment are provided in Table D.1.

PCR fragments were generated using 100 ng of genomic DNA as a template with primers at a concentration of 0.2 - 0.5 µM in a 50-µl reaction volume. A high-fidelity enzyme, Phusion™ High-Fidelity DNA Polymerase in GC buffer was used for all PCRs. PCR conditions were optimized for successful amplification of the targeted fragments (Table D.1), and two independent PCR reactions for each amplified fragment were performed. A no-template control and DNA from the control substance were also run to verify the purity of the reagents and the specificity of the PCR fragment. PCR products were separated on an agarose gel and visualized under ultraviolet (UV) light.

Table D.1. PCR Amplification Conditions

Cycle	Thermal Cycle Conditions[a] for PCR Fragments Generated from DAS1131 Maize							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1x	98ºC 1'	98ºC 30"	98ºC 1'	98ºC 30"	98ºC 30"	98ºC 1'	98ºC 30"	98ºC 1'
30x	98ºC 15" 60ºC 15" 72°C 1'	98ºC 10" 60ºC 10" 72°C 2'10"	98ºC 10" 60ºC 10" 72°C 2'	98ºC 10" 58ºC 10" 72°C 1'15"	98ºC 10" 62ºC 10" 72°C 1'15"	98ºC 15" 55ºC 15" 72°C 1'	98ºC 10" 58ºC 10" 72°C 1'15"	98ºC 15" 64ºC 15" 72°C 1'
1x	72ºC 10'	72ºC 10'	72ºC 10'	72ºC 3'	72ºC 10'	72ºC 10'	72ºC 3'	72ºC 10'
	4°C ∞	4°C ∞	4°C ∞	4°C ∞	4°C ∞	4°C ∞	4°C ∞	4°C ∞
Primer Conc.[b]	0.2 µM	0.5 µM	0.2 µM	0.4 µM	0.5 µM	0.2 µM	0.4 µM	0.2 µM

[a] Cycle time is indicated in minutes (') and seconds (").
[b] Primer concentration

Cloning of PCR Products and DNA Isolation

PCR products from two independent PCR reactions for each PCR fragment were separately cloned into pCR™ Blunt II TOPO® vectors using Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (Invitrogen).

At least three colonies from each transformation were chosen for plasmid DNA isolation. Plasmid DNA was isolated from the resulting bacterial culture using QIAprep Spin Miniprep Kit (Qiagen) and digested with restriction enzymes to confirm the presence of the cloned insert before sequencing. The purified plasmid DNA was quantified using a spectrophotometer.

Sanger DNA Sequencing

Six positive clones (three from each of the two independent PCR reactions) for each PCR fragment were sequenced by Sanger sequencing using M13 forward and reverse primers (except Fragment **C** which used T7 and M13 reverse primers) and multiple internal sequencing primers. The overlapping PCR fragments of the DAS1131 insert and flanking genomic regions were sequenced in both forward and reverse directions to cover every nucleotide by Sanger sequencing (Eurofins).

Sequencher® Version 5.1 software (Gene Codes Corporation) was used to analyze and assemble the sequences with default parameters. Low-quality data and vector sequences were trimmed from the 5' and 3' ends of each trace file when necessary. The sequences from the six clones were used to determine the consensus sequence for each PCR fragment. All reads were manually reviewed, and any ambiguous nucleotide was visually verified from the original chromatograms and compared with sequence reads from the other clones to make a final base call.

The final consensus sequence for the DAS1131 insert and flanking genomic regions was generated by combining the overlapping individual consensus sequences of the PCR fragments. The final consensus sequence determined for DAS1131 maize was compared with the sequence of the T-DNA of plasmid PHP88492.

APPENDIX E. METHODS FOR CHARACTERIZATION OF CRY1DA2 PROTEIN

Test Materials

Plant-Derived Cry1Da2 *Protein*: The Cry1Da2 protein was isolated from DAS1131 maize leaf 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) from plants grown at a field location in Johnston, IA, USA. The tissue was lyophilized, homogenized and stored at ≤ -50 °C. The Cry1Da2 protein was extracted from lyophilized maize tissue by homogenization with a Waring blender using chilled phosphate-buffered saline containing polysorbate 20 (PBST) extraction buffer (20-30 ml buffer per g tissue). The sample extract was clarified by filtration and centrifugation prior to purification by immunoaffinity chromatography. The immunoaffinity column was prepared by coupling a Cry1Da2 monoclonal antibody (DA447.788.181; Dow AgroSciences) to AminoLink Plus Coupling Resin. The Cry1Da2 protein was eluted off the column using ImmunoPure IgG Elution buffer. Elutions 2 and 3 from the immunoaffinity purification column were collected separately and combined for concentration using a centrifugal concentrator (30K Vivaspin; Sartorius) until the volume was reduced to approximately 200 µl. Following initial concentration, 500 µl of 50 mM Tris buffer, pH 8, was added to the concentrated sample and the sample was spun again until the volume was reduced to approximately 200 µl. 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 sample in the concentrator. After vortexing, 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 (-80 °C freezer unit).

Microbially Derived Cry1Da2 Protein: The Cry1Da2 protein was produced at Fraunhofer IME for Dow AgroSciences, LLC. using a microbial expression system. To produce protein with an intact N-terminus, two intended amino acid changes (K19Q and R27Q) were made. Both the lysine at position 19 and the arginine at position 27 were changed to glutamine. The protein was expressed as the Cry1Da2 protein with a crystal forming domain in a protein expression system through fermentation and purified from the cell lysate using inclusion body washing techniques. The inclusion bodies were then solubilized and digested with trypsin. After trypsin digestion to remove the crystal forming domain, the Cry1Da2 protein was purified using anion exchange chromatography prior to diafiltration to concentrate the protein and exchange the buffer into 10 mM CAPS pH 11.0. After lyophilization and mixing, a lot number was assigned (TSN-318947).

SDS-PAGE Analysis

The maize-derived Cry1Da2 protein samples were diluted as applicable, re-heated for 5 minutes at 90-100 °C, and then loaded into 4-12% Bis-Tris gels. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also loaded into each gel to provide a visual verification that migration was within the expected range of the predicted molecular weight. In addition, the microbially derived Cry1Da2 protein was diluted to approximately the same

concentration as the maize-derived protein, re-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, western blot analysis, protein glycosylation analysis, or sample preparation for N-terminal amino acid sequencing and peptide mapping.

For Coomassie staining of maize and microbially derived samples, gels were washed with water three times for 5 minutes each and were 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 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, the resulting gel was assembled into a 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 PBST containing 5% weight/volume (w/v) non-fat dry milk for 60 minutes at ambient laboratory temperature. Before 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 with a Cry1Da2 polyclonal antibody TG5072014 (Dow AgroSciences) diluted 1:20,000 in PBST containing 1% w/v non-fat dry milk at 2-8 °C overnight. Following primary antibody incubation, the membrane was washed in PBST four times for 5 minutes each wash. The membrane was incubated with a secondary antibody (anti-rabbit IgG, horseradish peroxidase conjugate; Promega Corporation) diluted in PBST 1:20,000 containing 1% w/v non-fat dry milk for 60 minutes at ambient laboratory temperature. The membrane was washed with PBST four 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 by Mass Spectrometry

Following SDS-PAGE, Coomassie staining, and imaging of the gels, the Cry1Da2 protein bands at the expected molecular weight of Cry1Da2 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 an ACQUITY UPLC (Waters Corporation) fitted with a UPLC column (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 MS Data Converter (Beta 1.3) to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software) and match peptides from the expected Cry1Da2 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 software.

N-Terminal Amino Acid Sequencing Analysis

Following SDS-PAGE, Coomassie staining, and gel imaging using the methods 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-P PVDF membrane was briefly wetted in 100% methanol, 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 transfer of the Cry1Da2 protein, the membrane was washed with water three times for 5 minutes each, stained with GelCode Blue stain reagent for 5 minutes, and then destained with water to visualize a protein band at the expected molecular weight of Cry1Da2 protein. A band containing the Cry1Da2 protein was excised and stored frozen at ≤ -5 °C.

The band 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 with applicable adjustments.

Glycoprotein Analysis

A Pierce Glycoprotein Staining Kit was used to determine whether the Cry1Da2 protein was glycosylated. The Cry1Da2 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 at least once for 5 minutes and then washed in water at least 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 washed with water at least once for 5 minutes to visualize all protein bands. The image of the GelCode stained gel was then captured electronically.

Bioactivity Bioassay

The biological activity of the Cry1Da2 protein was evaluated by conducting a 7-day bioassay using *Spodoptera frugiperda* (fall armyworm; Lepidoptera: Noctuidae), a species sensitive to the Cry1Da2 protein. Eggs were obtained from [redacted] and their identity was recorded by study personnel.

The *S. frugiperda* bioassay utilized a generalized randomized block design containing four blocks. Each block consisted of a 12-well bioassay plate and contained five replicates from each of the following treatments for a target of 20 individuals per treatment:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 50 ng Cry1Da2 protein per mg diet wet weight)

A Cry1Da2 protein stock solution was prepared by solubilizing 5.0 mg of test substance in 1.0 ml of ultrapure water. To prepare the test dosing solution, the stock solution was diluted in ultrapure water to a concentration of 66.7 ng/µl. The bioassay control dosing solution consisted of ultrapure water. Dosing solutions were prepared on Day 0 of the *S. frugiperda* bioassay and maintained chilled on wet ice until use. The carrier for the *S. frugiperda* bioassay consisted of Stonefly Heliothis diet. Each dosing solution was combined with carrier at a 3:1 ratio (i.e., 3 ml of dosing solution to 1 g carrier) to generate Treatments 1 and 2.

S. frugiperda eggs were incubated in an environmental chamber until the eggs hatched. Neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 1 ml (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of the bioassay plates. One *S. frugiperda* neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark for 7 days. After 7 days, the bioassay was complete, mortality was assessed, and surviving larvae were individually weighed.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 30% for the bioassay control diet (Treatment 1) group. The bioassay met the acceptability criteria.

Thermolability Analysis

The test substance consisted of Cry1Da2 protein solubilized from a lyophilized powder (lot number TSN318947). The carrier consisted of Stonefly Heliothis diet. The bioassay control dosing solution used to prepare Treatment 1 consisted of ultrapure (American Society for Testing and Materials [ASTM] Type 1) water. The bulk dosing solution used to prepare Treatments 2-6 consisted of aliquots of the test substance diluted in ultrapure water to achieve the targeted concentration in Treatments 2-6. The dosing solution aliquots used to prepare Treatments 3-6 were incubated for 30-35 minutes at several targeted temperatures.

The test system was *Spodoptera frugiperda* (fall armyworm; Lepidoptera: Noctuidae). The test system was chosen because *S. frugiperda* is an insect sensitive to the Cry1Da2 protein. *S. frugiperda* larvae were exposed via oral ingestion to one of the following six treatments:

- Treatment 1: Bioassay Control Diet containing ultrapure water
- Treatment 2: Control Diet containing the unheated Cry1Da2 protein dosing solution
- Treatment 3: Test Diet containing the Cry1Da2 protein dosing solution incubated at 25 °C
- Treatment 4: Test Diet containing the Cry1Da2 protein dosing solution incubated at 50 °C
- Treatment 5: Test Diet containing the Cry1Da2 protein dosing solution incubated at 75 °C
- Treatment 6: Test Diet containing the Cry1Da2 protein dosing solution incubated at 95 °C

Dosing solutions were prepared on the day of diet preparation for the *Spodoptera frugiperda* bioassay. The unheated control diet and each test diet contained a targeted concentration of 16 ng Cry1Da2 protein per mg diet wet weight. To generate the bulk Cry1Da2 protein dosing solution for Treatments 2-6, the test substance was thawed under chilled conditions and then diluted in ultrapure water to the appropriate Cry1Da2 protein concentration to achieve the targeted concentration in the test diets. Aliquots were dispensed into Eppendorf Protein LoBind tubes for heat treatment for 30-35 minutes using a heat block set to obtain temperatures (± 5 °C) of 25 °C, 50 °C, 75 °C, and 95 °C. One vial was left chilled (2-8 °C or on wet ice) as an unheated control treatment. Each dosing solution was mixed with carrier in a 3:1 ratio (i.e., 3 ml of dosing solution to 1 g of carrier). Treatments were arranged in a generalized randomized block design with a total of 10 blocks. Each block consisted of a 12-well bioassay plate and contained 2 replicates from each treatment. On Day 0, each treatment was provided to a target of 20 *S. frugiperda* individuals. Approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into wells of the bioassay plates. One *S. frugiperda* neonate was placed in each well containing diet. Each bioassay plate was sealed with heat-sealing film, a small hole was poked over each well to allow for ventilation, and the plates were placed in an environmental chamber. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark. Larvae were refed on Day 4 and missing or dead larvae were recorded. After 7 days, the bioassay was complete, final mortality was assessed, and surviving organisms were individually weighed.

The bioassay acceptability criteria were as follows: The bioassay may be terminated and repeated if the combined number of dead and missing organisms is greater than 20% for the bioassay control diet (Treatment 1) group. The bioassay may be terminated and repeated if the mortality of the unheated control diet (Treatment 2) group does not exceed 80%. The *S. frugiperda* bioassay met both acceptability criteria. An enzyme-linked immunosorbent assay (ELISA) was used to assess the homogeneity of the Cry1Da2 protein in Treatment 2 and concentration of the Cry1Da2 protein dosing solutions. The absence of Cry1Da2 protein in the bioassay control dosing solutions was also assessed. Bias in the *S. frugiperda* bioassay was controlled through the randomization of treatments within blocks and the use of one or more control diets. Bias in the characterization portion of the study was controlled through the use of replicate testing and appropriate assay controls.

Statistical Analysis

Statistical analyses of data were conducted using SAS software, Version 9.4 (SAS Institute, Inc.). The response variable of interest was mortality. Statistical comparisons were made between mortality of *S. frugiperda* fed diet containing heat-treated Cry1Da2 protein (Treatments 3, 4, 5 and 6) and that of *S. frugiperda* fed the unheated Cry1Da2 protein control diet (Treatment 2).

Statistical analysis was conducted using Fisher's exact test to determine if the mortality rate of *S. frugiperda* fed diets containing the heat-treated Cry1Da2 protein (m_T) was lower than the mortality rate of those fed the unheated Cry1Da2 protein control diet (m_C). The corresponding hypothesis test was

$$H_0: m_T - m_C = 0 \quad vs. \quad H_a: m_T - m_C < 0.$$

A significant difference was established if the P-value was < 0.05. SAS PROC MULTTEST was utilized to conduct the Fisher's exact test.

Digestibility in Simulated Gastric Fluid (SGF)

Test and control solutions were prepared as follows:

- The gastric control solution was prepared fresh on the day of use and was comprised of 0.2% weight per volume (w/v) NaCl in 0.7% volume per volume (v/v) HCl with a pH of ~1.2.
- The pepsin digestion solution, referred to as simulated gastric fluid (SGF), was prepared fresh on the day of use by dissolving pepsin (Sigma-Aldrich) into gastric control solution. The SGF was prepared so that the pepsin to protein ratio of the final digestion mixture was 10 units of pepsin per µg of test substance.
- The Cry1Da2 protein stock solution was prepared fresh on the day of use by solubilizing a 5.0 mg sub-sample of Cry1Da2 protein powder (PCF-0056) in 0.65 ml of ASTM [American Society for Testing and Materials] Type I ultrapure water to a target Cry1Da2 protein concentration of 5 mg/ml.
- To prepare the stock solutions for each of the control proteins (BSA and β-lactoglobulin), a sub-sample of 5.0 mg powder was weighed into an individual tube and solubilized by adding 1 ml of water (for a final protein concentration of 5 mg/ml).
- The final concentration of the protein and pepsin in the SGF reaction mixture was 0.25 mg/ml Cry1Da2 protein and 2500 units/ml pepsin.

SGF solution (1,900 µl) was dispensed into a 7-ml glass vial and placed in a 37 °C water bath for 2-5 minutes prior to the addition of 100 µl of Cry1Da2 protein stock solution at Time 0. The digestion reaction mixture was mixed constantly using a stir bar and a submersible magnetic stirrer.

A 120-µl sub-sample of the Cry1Da2 protein digestion reaction mixture was removed from the vial at the following analytical time points (± 10 seconds): 0.5, 1, 2, 5, 10, 20, and 60 minutes. The sub-samples were inactivated by adding them to pre-labeled tubes containing 139 µl of a pre-mixed sample stop solution (consisting of 48 µl of 200 mM sodium carbonate, 65 µl NuPAGE 4X LDS sample buffer, and 26 µl NuPAGE 10X sample reducing agent) and heating to 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

To prepare control digestion samples at 1 and 60 minutes, a 114-µl sample of the appropriate digestion solution (see table) was pre-warmed in a 37 °C water bath for 2-5 minutes prior to adding 6 µl of the Cry1Da2 protein stock solution, control protein stock solution, or water. The tubes were incubated in the water bath for the allotted time and then inactivated by mixing with 139 µl of the pre-mixed sample stop solution.

The Time 0 control reaction mixtures were prepared by first neutralizing 114 µl of the appropriate digestion solution with 139 µl of the pre-mixed sample stop solution, and then adding 6 µl of the Cry1Da2 protein stock solution, control protein stock solution, or water to the appropriate tube and mixing.

Control digestion samples included in the SGF assay are provided in Table E.1. Following digestion and inactivation, all control reaction mixtures were heated at 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

Table E.1. Control Samples for Simulated Gastric Fluid (SGF) Digestibility Analysis

Protein	Digestion Solution	Digestion Time (min)		
		0	1	60
None (Water), {SGF Control}	SGF	X	--	X
BSA	SGF	X	X	X
β-lactoglobulin	SGF	X	X	X
Cry1Da2	SGF	X	--	--
Cry1Da2	None (Water)	X	--	X
Cry1Da2	Gastric Control Solution (No Pepsin)	--	--	X

SDS-PAGE Analysis

The Cry1Da2 protein digestion time-course samples and control digestion samples were removed from frozen storage, heated at 90-100 °C for 5 minutes, and loaded (10 µl/well) into 4-12% Bis-Tris gels for SDS-PAGE analysis. To demonstrate the sensitivity of the SDS-PAGE gel and western blot analyses, an aliquot of the Cry1Da2 protein in SGF (Time 0) sample was loaded into the gel at a 1:20 dilution (58 ng Cry1Da2 protein) for protein staining, and at a 1:100 dilution (11.6 ng Cry1Da2 protein) for the western blot. Pre-stained protein molecular weight markers (Precision Plus Dual Xtra Standards) were also loaded into the gels to provide a visual estimate of molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES SDS running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes for use in protein staining or western blot analyses. For protein staining, the gels were washed with water three times for 5 minutes each and stained with GelCode Blue Stain Reagent for 67 minutes. Following staining, the gels were destained with water four times for a minimum of 5 minutes each until the gel background was clear. Proteins were stained as blue-colored bands on the gels. The gel image was captured electronically using a ChemiDoc MP (Bio-Rad) imaging system.

Western Blot Analysis

The Cry1Da2 protein digestion time-course samples were also analyzed by western blot. Following SDS-PAGE, the gel intended for western blot analysis 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 phosphate-buffered saline containing polysorbate 20 (PBST) containing 5% (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 at least 1 minute each to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with a Cry1Da2 polyclonal antibody NB201104010 (Pioneer Hi-Bred International, Inc.) diluted 1:100,000 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. The membrane was incubated for 60 minutes at ambient laboratory temperature with a secondary antibody (anti-rabbit IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:100,000 in PBST containing 1% (w/v) non-fat dry milk. The membrane was washed in PBST four times for 5 minutes each. 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.

Digestibility in Simulated Intestinal Fluid (SIF)

Test and control solutions were prepared as follows:

- The pancreatin digestion solution, referred to as simulated intestinal fluid (SIF), was prepared fresh on the day of use by dissolving 26.4 mg of pancreatin (Sigma-Aldrich) into 5 ml of intestinal control solution (I-Con 1X buffer) to a final concentration of 0.5% weight per volume (w/v) pancreatin and 50 mM KH_2PO_4, pH 7.5.
- The Cry1Da2 protein stock solution was prepared fresh on the day of use by solubilizing a 10.0-mg sub-sample of Cry1Da2 protein powder (PCF-0056) in 1.3 ml of ASTM [American Society for Testing and Materials] Type I ultrapure water to a target protein concentration of 5.0 mg/ml.
- To prepare the stock solutions for each of the control proteins (BSA and β-lactoglobulin), a 5.0-mg sub-sample of powder was weighed into an individual tube for each control and solubilized by adding 1.0 ml of water (to a target protein concentration of 5.0 mg/ml).
- The final concentration of the protein and pancreatin in the SIF reaction mixture was 0.25 mg/ml Cry1Da2 protein and 0.5% (w/v) pancreatin.

SIF solution (1900 µl) was dispensed into a 7-ml glass vial and placed in a 37 °C water bath for 2-5 minutes prior to the addition of 100 µl of Cry1Da2 protein test substance at Time 0. The digestion reaction mixture was mixed constantly using a stir bar and a submersible magnetic stirrer.

A 120-µl sub-sample of the Cry1Da2 protein digestion reaction mixture was removed from the vial at the following analytical time points (± 10 seconds): 0.5, 1, 2, 5, 10, 20, 30, and 60 minutes. The sub-samples were inactivated by adding them to pre-labeled tubes containing 64 µl of pre-mixed sample solution (consisting of 46 µl NuPAGE 4X LDS Sample Buffer and 18 µl

NuPAGE 10X Sample Reducing Agent) and heating to 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

To prepare control digestion samples at 1 and 60 minutes, a 114-µl sample of the appropriate digestion solution (see table) was pre-warmed in a 37 °C water bath for 2-5 minutes prior to adding 6 µl of the Cry1Da2 protein test substance, control protein stock solution, or water. The tubes were incubated in the water bath for the allotted time and then inactivated by mixing with 64 µl of the pre-mixed sample solution.

The Time 0 control reaction mixtures were prepared by first neutralizing 114 µl of the appropriate digestion solution with 64 µl of the pre-mixed sample solution, and then adding 6 µl of the Cry1Da2 protein test substance, protein stock solution, or water to the appropriate tube and mixing.

Following digestion and inactivation, all control reaction mixtures were heated at 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

Control digestion samples included in the SIF assay are provided in Table E.2.

Table E.2. Control Samples for Simulated Intestinal Fluid (SIF) Digestibility Analysis

Protein	Digestion Solution	Digestion Time (min)		
		0	1	60
None (Water), {SIF Control}	SIF	X	--	X
BSA	SIF	X	X	X
β-lactoglobulin	SIF	X	X	X
Cry1Da2	SIF	X	--	--
Cry1Da2	None (Water)	X	--	X
Cry1Da2	Intestinal Control Solution (No Pancreatin)	--	--	X

SDS-PAGE Analysis

The Cry1Da2 protein digestion time-course samples and control samples were removed from frozen storage, heated at 90-100 °C for 5 minutes, and loaded (10 µl/well) into 4-12% Bis-Tris gels for SDS-PAGE analysis. Pre-stained protein molecular weight markers (Precision Plus Dual Xtra Standards) were also loaded into each gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES SDS running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes for use in protein staining or western blot analyses. For protein staining, the gels were washed three times for 5 minutes each with water and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, the gels were destained with water four times for a minimum of 5 minutes each until the gel background was clear. Proteins were stained as blue-colored bands on the gels. The gel image was captured electronically using a ChemiDoc MP (Bio-Rad) imaging system.

Western Blot Analysis

The Cry1Da2 protein digestion time-course samples were also analyzed by western blot. Following SDS-PAGE, one of the resulting gels 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 phosphate-buffered saline containing polysorbate 20 (PBST) containing 5% (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 to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with an Cry1Da2 polyclonal antibody NB 201104010 (Pioneer Hi-Bred International, Inc.) diluted 1:100,000 in PBST containing 1% (w/v) non-fat dry milk. Following primary antibody incubation, the membrane was washed in PBST four times for 5 minutes each. The membrane was incubated for 60 minutes at ambient laboratory temperature with a secondary antibody (anti-rabbit IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:100,000 in PBST containing 1% (w/v) non-fat dry milk. The membrane was washed in PBST four times for at least 5 minutes each. 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.

Sequential Digestibility Analysis with Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

Test solutions were prepared as follows:

- A concentrated (i.e., 2X) pepsin digestion solution, referred to as simulated gastric fluid (SGF), was prepared fresh on the day of use by solubilizing pepsin (Sigma-Aldrich) in a previously prepared 2X gastric control solution. The final concentration of gastric control solution in SGF was 0.2% weight per volume (w/v) NaCl and 0.7% volume per volume (v/v) HCl; pH ~1.2. The SGF was prepared so that the pepsin to protein ratio of the final digestion mixture was 10 units of pepsin per µg of test protein.
- A concentrated (i.e., 2.5X) pancreatin digestion solution, referred to as simulated intestinal fluid (SIF), was prepared fresh on the day of use by solubilizing pancreatin (Sigma-Aldrich) in 2.5X intestinal control solution (2.5X I-Con). The final concentration of intestinal control solution in SIF was 50 mM KH_2PO_4, with a pH of ~7.5. Pancreatin content in SIF was adjusted so that there was approximately 0.5% (w/v) pancreatin in the final digestion reaction mixture.
- The pre-mixed sample stop solutions used to inactivate samples were prepared fresh on the day of use. The solution for SGF reactions was prepared by mixing 240 µl of 200 mM Na2CO3, 325 µl NuPAGE 4X LDS Sample Buffer, and 130 µl NuPAGE 10X Sample Reducing Agent. The solution for SIF reactions was prepared by mixing 1150 µl NuPAGE 4X LDS Sample Buffer and 450 µl NuPAGE 10X Sample Reducing Agent.
- The Cry1Da2 protein stock solution was prepared fresh on the day of use by solubilizing a 10.0 mg sub-sample of Cry1Da2 protein powder (PCF-0056) in 1.3 ml of ASTM [American Society for Testing and Materials] Type I ultrapure water to a target protein concentration of 5 mg/ml.

In Vitro Pepsin Digestion

Cry1Da2 Protein in SGF 1 Minutes Sample for Sequential Digestion: An aliquot (1 ml) of the 2X SGF solution and 800 µl water were dispensed into a 7-ml glass vial and pre-warmed in the 37 °C water bath for 2-5 minutes prior to addition of 200 µl of the Cry1Da2 protein test substance. The SGF digestion reaction mixture was incubated and mixed constantly using a stir bar and submersible stir plate for 1 minutes (± 10 seconds) after adding the Cry1Da2 protein test substance. At the end of the time period, a 1.5-ml sample of the Cry1Da2 SGF digestion reaction mixture was transferred to a separate vial and inactivated by neutralization with 0.3 ml of 0.5 N NaOH. This sample was used for the sequential SIF digestion.

Cry1Da2 Protein in SGF 1 Minutes: A 120-µl control sample (Cry1Da2 in SGF 1 minutes) was taken out from the SGF digestion reaction mixture at the end of 1 minutes (± 10 seconds) and inactivated by neutralization with 139 µl of pre-mixed SGF sample stop solution. The neutralized sample was heated for 5 minutes at 90-100 °C prior to frozen storage (-20 °C freezer unit).

Cry1Da2 Protein in SGF Time 0: A control sample (Cry1Da2 in SGF Time 0) was prepared by first inactivating 60 µl of 2X SGF and 49 µl water in 139 µl of pre-mixed SGF sample stop solution and then adding 12 µl of Cry1Da2 protein test substance to the neutralized SGF. The neutralized sample was heated for 5 minutes at 90-100 °C prior to frozen storage (-20 °C freezer unit).

SGF-Only 1 Minutes Incubation: An SGF-only control sample without Cry1Da2 protein test substance (SGF Control 1minute) was prepared by mixing 60 µl 2X SGF and 49 µl water in a tube and pre-warming at 37 °C for 2-5 minutes. Following the addition of 12 µl of water, the tube was incubated in a 37 °C water bath for 1 minutes (± 10 seconds). After incubation, the sample was inactivated by neutralization with 139 µl of pre-mixed SGF sample stop solution. The neutralized sample was heated for 5 minutes at 90-100 °C prior to frozen storage (-20 °C freezer unit).

Sequential Pancreatin Digestion

Cry1Da2 Protein in SGF 1 Minutes, SIF 0.5-30 Minutes: For the sequential SIF digestion time course, a 1.2-ml sample of the neutralized Cry1Da2 SGF digestion reaction mixture was dispensed into a 7-ml glass vial and placed in a 37 °C water bath for 2-5 minutes prior to addition of 800 µl 2.5X SIF solution. The SIF digestion reaction mixture was mixed constantly using a stir bar and a submersible stir plate.

A 120-µl sub-sample of the SIF digestion reaction mixture was removed from the vial at each of the following analytical time points (± 10 seconds): 0.5, 1, 2, 5, 10, 20 and 30 minutes. Each sub-sample was neutralized by adding it to a pre-labeled tube containing 64 µl of pre-mixed SIF sample stop solution. The neutralized samples were inactivated by heating at 90-100 °C for 5 minutes.

Cry1Da2 Protein in SGF 1 Minutes, SIF Time 0: A SIF control sample (Cry1Da2 1 minutes SGF Time 0 SIF) was prepared by mixing 48 µl 2.5X SIF with 64 µl of pre-mixed SIF sample stop solution and then heating for 5 minutes at 90-100 °C. A sub-sample (72 µl) of the neutralized Cry1Da2 SGF digestion reaction mixture was added to the heat-inactivated SIF control sample and then heated again for 5 minutes at 90-100 °C.

After neutralization and heating, all SIF reaction samples were stored frozen (-20 °C freezer unit).

SDS-PAGE Analysis

The digestion samples were removed from frozen storage, heated at 90-100 °C for 5 minutes, and loaded (10 µl/well) into a 4-12% Bis-Tris gel for SDS-PAGE analysis. Pre-stained protein molecular weight markers (Precision Plus Dual Xtra Standards) were also loaded into the gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gel was removed from the gel cassette and washed three times for 5 minutes each with water and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, the gel was destained with water four times for a minimum of 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.

APPENDIX E.2 CHARACTERIZATION OF CRY1DA2 (PCF-0056) PROTEIN DERIVED FROM A MICROBIAL EXPRESSION SYSTEM

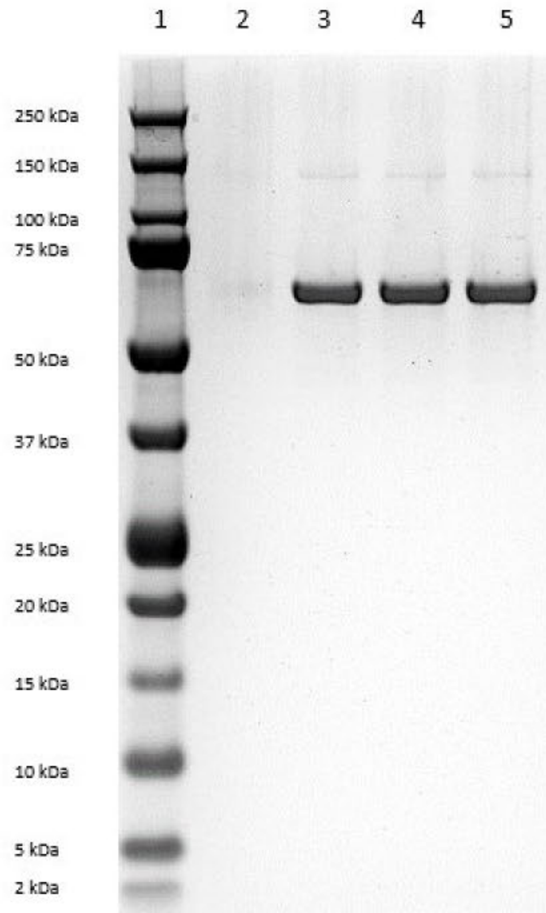
In order to have sufficient amounts of purified Cry1Da2 protein for the multiple studies required to assess its safety, the Cry1Da2 protein was expressed in a [redacted] protein expression system. The Cry1Da2 protein was produced at [redacted] for Pioneer Hi-Bred International, Inc. The protein was expressed in a [redacted] protein expression system through fermentation and purified from the cell lysate using inclusion body washing techniques. The inclusion bodies were then solubilized and digested with trypsin. After trypsin digestion of the Cry1Da2 protein, it was purified using anion exchange chromatography prior to diafiltration to concentrate the protein and exchange the buffer into 10 mM CAPS, pH 11.0. The Cry1Da2 protein was lyophilized, mixed, and assigned a lot number (PCF-0056).

The Cry1Da2 protein was characterized for concentration, purity, molecular weight, immunoreactivity, lack of glycosylation, amino acid sequence, molecular mass, and bioactivity. The methods used include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, western blot analysis, protein glycosylation analysis, liquid chromatography-mass spectrometry (LC-MS), N-terminal amino acid sequencing, and a bioactivity assay.

SDS-PAGE Analysis

The microbially derived Cry1Da2 protein migrated as a predominant band consistent with the expected molecular weight of approximately 68 kilodaltons (kDa) (Figure E2.1). Densitometry analysis indicated that the purity of the Cry1Da2 protein was a predominant band and is therefore reported as > 95% on a total protein basis. The concentration of the test substance was determined to be 0.65 milligrams (mg) of protein per mg of lyophilized powder.

Additional details regarding SDS-PAGE analytical methods are provided in Appendix E.3.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS Sample Buffer Blank
3	Microbially Derived Cry1Da2 Protein
4	Microbially Derived Cry1Da2 Protein
5	Microbially Derived Cry1Da2 Protein

Note: kilodalton (kDa) and microgram (µg).

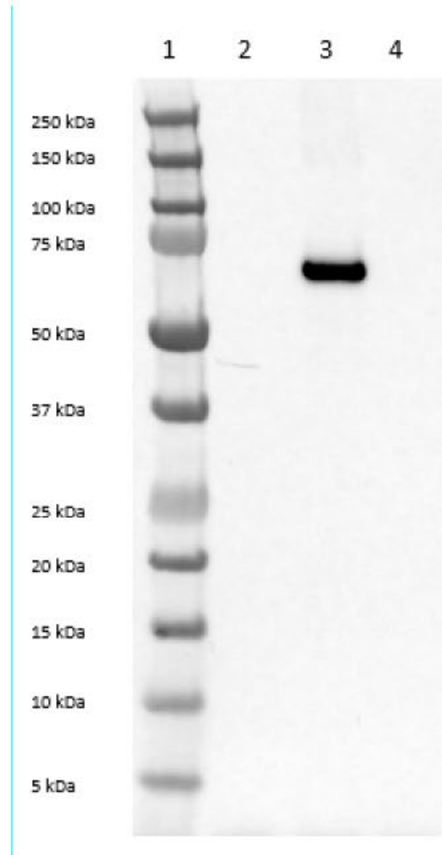
[a] Molecular weight markers were included to provide a visual estimate that migration was within the expected range of the predicted molecular weight.

Figure E2.1. SDS-PAGE Analysis of Microbially Derived Cry1Da2 Protein

Western Blot Analysis

Western blot analysis demonstrated that the Cry1Da2 protein was immunoreactive to a Cry1Da2 polyclonal antibody and visible as a predominant band consistent with the expected molecular weight of approximately 68 kDa (Figure E2.2).

Additional details regarding Western blot analytical methods are provided in Appendix E.3.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[a]
2	1X LDS Sample Buffer Blank
3	Microbially Derived Cry1Da2 Protein (5 ng)
4	1X LDS Sample Buffer Blank

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

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

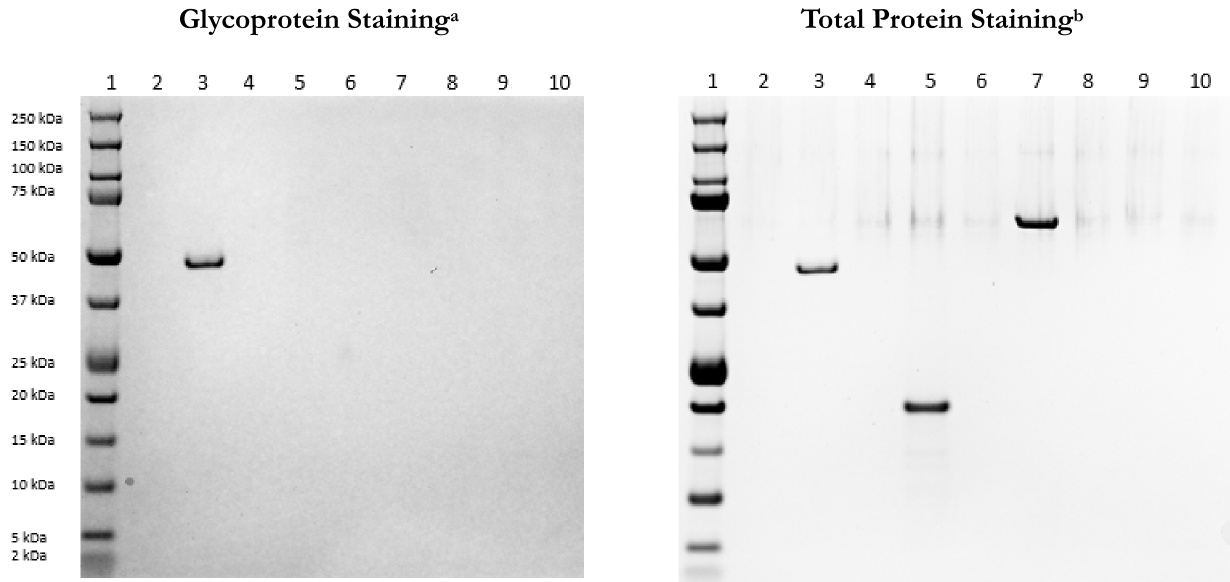
Figure E2.2. Western Blot Analysis of Microbially Derived Cry1Da2 Protein

Protein Glycosylation Analysis

The microbially derived Cry1Da2 protein was analyzed by SDS-PAGE. The gel also included a positive control (horseradish peroxidase) and negative control (soybean trypsin inhibitor). The gel was then stained using a Pierce Glycoprotein Staining Kit to visualize any glycoproteins. The gel was imaged and then stained with GelCode Blue stain reagent to visualize all protein bands.

Glycosylation was not detected for the microbially derived Cry1Da2 protein (Figure E2.3). 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 glycosylation analytical methods are provided in Appendix E.3.



Lane	Sample Identification
1	Pre-stained Protein Molecular Weight Marker[c]
2	1X LDS Sample Buffer Blank
3	Horseradish Peroxidase (1 µg)
4	1X LDS Sample Buffer Blank
5	Soybean Trypsin Inhibitor (1 µg)
6	1X LDS Sample Buffer Blank
7	Microbially Derived Cry1Da2 Protein (1 µg)
8	1X LDS Sample Buffer Blank
9	1X LDS Sample Buffer Blank
10	1X LDS Sample Buffer Blank

Note: kilodalton (kDa) and microgram (µg).

[a] Gel was stained with glycoprotein staining reagent.

[b] Gel was stained with glycoprotein staining reagent followed by staining with Coomassie Blue Reagent for total proteins.

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

Figure E2.3. Glycosylation Analysis of Microbially Derived Cry1Da2 Protein

Mass Spectrometry Peptide Mapping Analysis

The microbially derived Cry1Da2 was analyzed by SDS-PAGE. Protein bands were stained with Coomassie stain reagent, and bands containing the Cry1Da2 protein were excised for each sample.

The excised Cry1Da2 protein bands were digested with trypsin or chymotrypsin. Digested samples were analyzed with ultra-performance liquid chromatography-mass spectrometry (LC-MS). The resulting MS data was used to search and match the peptides from the expected Cry1Da2 protein sequence, and the combined sequence coverage was calculated. The identified tryptic and chymotryptic peptides for microbially derived Cry1Da2 protein are shown in Tables E2.1 and E2.2, respectively. The combined sequence coverage accounts for 86.2% (520/603) of the expected Cry1Da2 amino acid sequence (Table E2.3.6 and Figure E2.4).

Additional details regarding peptide mapping analytical methods are provided in Appendix E.3.

Table E2.1. Identified Tryptic Peptides of Microbially Derived Cry1Da2 Protein Identified Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
87–92	763.3875	763.3864	IEEFAR
93–98	687.3671	687.3664	NQAISR
99–107	1035.5579	1035.56	LEGLSNLYK
112–118	881.3917	881.3919	AFSDWEK
112–126	1745.836	1745.8373	AFSDWEKDPTNPALR
119–126	882.4564	882.4559	DPTNPALR
119–130	1427.6836	1427.6827	DPTNPALREEMR
131–148	2079.0816	2079.0823	IQFNDMNSALITAIPLFR[c]
173–180	907.4398	907.44	DVSVFGER
181–191	1309.6017	1309.6051	WGYDTATINNR
192–215	2868.3436	2868.3501	YSDLTSLIHVYTNHCVDTYNQGLR
221–230	1311.6587	1311.6612	FLSDWIVYNR
253–264	1391.7402	1391.7409	TYPIQTATQLTR
316–328	1568.7514	1568.7524	YAYWGGHLVNSFR
329–337	975.5339	975.5349	TGTTTNLIR
338–343	691.3657	691.3653	SPLYGR
344–363	2170.1373	2170.1382	EGNTERPVTITASPSVPIFR
364–391	2994.4838	2994.4934	TLSYITGLDNSNPVAGIEGVEFQNTISR
392–396	665.3868	665.386	SIYRK
396–422	2841.3932	2841.3933	KSGPIDSFSELPPQDASVSPAIGYSHR
397–422	2713.2992	2713.2984	SGPIDSFSELPPQDASVSPAIGYSHR
423–431	1145.5665	1145.5652	LCHATFLER
437–447	1273.653	1273.6568	IAGTVFSWTHR
448–459	1230.5833	1230.584	SASPTNEVSPSR
460–467	983.5799	983.5804	ITQIPWVK
468–479	1153.6468	1153.6455	AHTLASGASVIK
468–491	2325.2455	2325.2441	AHTLASGASVIKGPGFTGGDILTR
480–491	1189.6064	1189.6092	GPGFTGGDILTR
480–501	2248.1255	2248.127	GPGFTGGDILTRNSMGELGTLR
492–501	1076.5279	1076.5284	NSMGELGTLR
502–507	679.366	679.3653	VTFTGR
502–515	1699.9033	1699.9046	VTFTGRLPQSYYIR
508–515	1038.5479	1038.5498	LPQSYYIR
518–524	779.3924	779.3926	YASVANR
530–542	1469.7207	1469.7191	YSQPPSYGISFPK
543–553	1176.5424	1176.5445	TMDAGEPLTSR
554–568	1724.8869	1724.8886	SFAHTTLFTPITFSR
569–585	2044.9756	2044.9742	AQEEFDLYIQSGVYIDR
586–603	2032.0295	2032.0364	IEFIPVTATLEAESDLER

[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 closest to the experimental mass.
[c] This peptide was modified by methionine oxidation (Oxidation-M).

Table E2.2. Identified Chymotryptic Peptides of Microbially Derived Cry1Da2 Protein Identified Using LC-MS Analysis

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
1–12	1508.6366	1508.6388	MEINNQNQCVPY
73-82	1188.6389	1188.6390	DIFLAQIEQL
76-90	1815.9718	1815.9730	LAQIEQLISQRIEEF
83–90	1020.5234	1020.5240	ISQRIEEF
91–102	1326.7387	1326.7368	ARNQAISRLEGL
114-133	2461.1716	2461.1696	SDWEKDPTNPALREEMRIQF
134–146	1371.7063	1371.7068	NDMNSALITAIPL
147–152	825.4136	825.4133	FRVQNY
148-157	1203.6600	1203.6611	RVQNYEVALL
158–166	963.5018	963.5025	SVYVQAANL
158-168	1213.6462	1213.6455	SVYVQAANLHL
161–168	864.4817	864.4817	VQAANLHL
161–171	1177.6821	1177.6819	VQAANLHLSIL
169–177	1034.5759	1034.5760	SILRDVSVF
178–183	766.3410	766.3398	GERWGY
178-192	1814.8330	1814.8340	GERWGYDTATINNRY
182–192	1286.5879	1286.5891	GYDTATINNRY
192-195	1601.7303	1601.7321	GYDTATINNRYSDL
184–192	1066.5032	1066.5043	DTATINNRY
184–195	1381.6467	1381.6474	DTATINNRYSDL
184-198	1682.8108	1682.8111	DTATINNRYSDLTSL
193-202	1146.5910	1146.5921	SDLTSLIHVY
196–202	831.4492	831.4491	TSLIHVY
203–210	1008.3959	1008.3971	TNHCVDTY
215-222	1045.6161	1045.6145	RRLEGRFL
218-225	1008.4669	1008.4665	EGRFLSDW
222-228	894.4489	894.4487	LSDWIVY
246–262	2040.0352	2040.0316	FPNYDIRTYPIQTATQL
250–262	1518.8037	1518.8042	DIRTYPIQTATQL
263-272	1251.6481	1251.6499	TREVYLDLPF
273–286	1522.7302	1522.7303	INENLSPAASYPTF
287–302	1711.8877	1711.8893	SAAESAIIRSPHLVDF
287–303	1824.9743	1824.9734	SAAESAIIRSPHLVDFL
307-316	1201.5959	1201.5979	TIYTDSLARY
319-327	1015.4867	1015.4876	WGGHLVNSF
320–327	829.4088	829.4083	GGHLVNSF
328–335	862.4514	862.4509	RTGTTTNL
328–340	1428.8061	1428.8049	RTGTTTNLIRSPL
336–341	747.4285	747.4279	IRSPLY
341–362	2390.2282	2390.2230	YGREGNTERPVTITASPSVPIF
366–385	2080.9991	2080.9953	SYITGLDNSNPVAGIEGVEF
368–385	1830.9007	1830.9000	ITGLDNSNPVAGIEGVEF
372–385	1446.6634	1446.6627	DNSNPVAGIEGVEF
386–394	1080.5545	1080.5564	QNTISRSIY
395–403	1005.5237	1005.5243	RKSGPIDSF
395–419	2617.3073	2617.3024	RKSGPIDSFSELPPQDASVSPAIGY
404–419	1629.7889	1629.7886	SELPPQDASVSPAIGY
420–428	1127.5319	1127.5294	SHRLCHATF
429–442	1514.8574	1514.8569	LERISGPRIAGTVF
430–442	1401.7743	1401.7728	ERISGPRIAGTVF
445–465	2363.1994	2363.1982	THRSASPTNEVSPSRITQIPW
466–471	667.4023	667.4017	VKAHTL
472–483	1089.5804	1089.5819	ASGASVIKGPGF
472–489	1645.8697	1645.8676	ASGASVIKGPGFTGGDIL
484-500	1733.8624	1733.8618	TGGDILTRNSMGELGTL
490–500	1177.5719	1177.5761	TRNSMGELGTL
498–504	792.4497	792.4494	GTLRVTF

Table E2.2. Identified Chymotryptic Peptides of Microbially Derived Cry1Da2 Protein by LC-MS Analysis (continued)

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
505–512	920.4711	920.4716	TGRLPQSY
505–513	1083.5331	1083.5349	TGRLPQSYY
517–528	1327.6674	1327.6633	RYASVANRSGTF
519–528	1008.4984	1008.4989	ASVANRSGTF
529–536	996.4658	996.4665	RYSQPPSY
529–550	2440.1777	2440.1733	RYSQPPSYGISFPKTMDAGEPL
537–550	1461.7173	1461.7174	GISFPKTMDAGEPL
556-566	1247.6543	1247.6550	AHTTLFTPITF
567-576	1256.5669	1256.5673	SRAQEEFDLY
583–588	791.4180	791.4177	IDRIEF
583–595	1486.8403	1486.8395	IDRIEFIPVTATL
589–603	1642.8406	1642.8414	IPVTATLEAESDLER
596–603	947.4189	947.4196	EAESDLER

[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 closest to the experimental mass.

Table E2.3. Combined Sequence Coverage of Identified Tryptic and Chymotryptic Peptides of Microbially Derived Cry1Da2 Protein Using LC-MS Analysis

Protease	% Coverage	Combined % Coverage
Trypsin	66	86.2
Chymotrypsin	80	

1 **MEINNQNQCV PY**NCLSNPQE IILGEEQLET GNTVADISLG LINFLYSNFV
51 PGGGFIVGLL ELIWGFIGPS QW**DIFLAQIE QLISQRIEEF ARNQAISRLE**
101 **GLSNLYK**VYV R**AFSDWEKDP TNPALREEMR IQFNDMNSAL ITAIPLFRVQ**
151 **NYEVALLSVY VQAANLHLSI LRDVSVFGER WGYDTATINN RYSDLTSLIH**
201 **VYTNHCVDTY NQGLRRLEGR FLSDWIVYNR** FRRQLTISVL DIVAF**FPNYD**
251 **IRTYPIQTAT QLTREVYLDL PFINENLSPA ASYPTFSAAE SAIIRSPHLV**
301 **DFL**NSF**TIYT DSLARYAYWG GHLVNSFRTG TTTNLIRSPL YGREGNTERP**
351 **VTITASPSVP IFRTLSYITG LDNSNPVAGI EGVEFQNTIS RSIYRKSGPI**
401 **DSFSELPPQD ASVSPAIGYS HRLCHATFLE RISGPRIAGT VFSWTHRSAS**
451 **PTNEVSPSRI TQIPWVKAHT LASGASVIKG PGFTGGDILT RNSMGELGTL**
501 **RVTFTGRLPQ SYYIR**F**RYAS VANRSGTFRY SQPPSYGISF PKTMDAGEPL**
551 **TSRSFAHTTL FTPITFSRAQ EEFDLYIQSG VYIDRIEFIP VTATLEAESD**
601 **LER**

Red bold type	Red type indicates microbially derived Cry1Da2 peptides identified using LC-MS analysis against the expected[a] microbially derived Cry1Da2 sequence
Amino acid residue abbreviations	alanine (A), cysteine (C), 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).

[a] The expected sequence refers to the translated sequence for the gene derived from the expression vector and used for production of the Cry1Da2 protein toxicology lot.

Figure E2.4. Identified Tryptic and Chymotryptic Peptide Amino Acid Sequence of Microbially Derived Cry1Da2 Protein by LC-MS Analysis

N-Terminal Amino Acid Sequence Analysis

N-terminal amino acid sequence analysis identified a sequence (MEINNQNQ_V) matching amino acid residues 1-8 and 10 of the expected protein sequence. The ninth amino acid in the sequence was cysteine; this amino acid was not determined as the analysis used is unable to identify cysteine residues.

Additional details regarding N-terminal amino acid sequence analytical methods are provided in Appendix E.3.

Bioactivity assay

The bioactivity of the microbially derived Cry1Da2protein was evaluated by conducting a 7-day bioassay using *Spodoptera frugiperda* (fall armyworm; Lepidoptera: Noctuidae), a species sensitive to the Cry1Da2 protein.

Bioactivity analysis demonstrated that the Cry1Da2 protein had insecticidal activity toward a target insect, *S. frugiperda*. The biological activity of the test diet containing 50 ng Cry1Da2 protein was demonstrated by 100% mortality of *S. frugiperda* fed the test diet (Table E2.4).

Additional details are provided in Appendix E.3.

Table E2.4. Microbially Derived Cry1Da2Protein Bioactivity Assay Using *Spodoptera frugiperda*

Treatment	Treatment Description	Protein Concentration (ng Cry1Da2/mg	Total Number of Observations	Total Number of Dead Organisms	Mortality (%)
A	Bioassay Control Diet	0	20	0	0
B	Test Diet	50	20	20	100

Note: The concentration of Cry1Da2 protein in Treatment 2 was based on the wet weight of the artificial diet. The summary of *Spodoptera frugiperda* mortality data consisted of the calculation of dead larvae divided by the total number of observed larvae at the end of the study and multiplied by 100.

APPENDIX E.3. METHODS FOR CHARACTERIZATION OF MICROBIALLY DERIVED CRY1DA2 PROTEIN (PCF-0056)

Test Materials

Microbially Derived Cry1Da2 Protein: Cry1Da2 protein was produced at [redacted] for Pioneer Hi-Bred International, Inc. The protein was expressed in a [redacted] protein expression system through fermentation and purified from the cell lysate using inclusion body washing techniques. The inclusion bodies were then solubilized and digested with trypsin. After trypsin digestion of the Cry1Da2 protein, it was purified using anion exchange chromatography prior to diafiltration to concentrate the protein and exchange the buffer into 10 mM CAPS, pH 11.0. The Cry1Da2 protein was lyophilized, mixed, and assigned a lot number (PCF-0056).

For concentration analysis, five samples of the Cry1Da2 protein test substance (~25 mg) were solubilized in 25mM CAPS buffer, pH 10.5, and diluted with 2X LDS/DTT solution (50% 4X NuPAGE LDS Sample Buffer, 20% 10X NuPAGE Sample Reducing Agent containing DTT, and 30% ASTM [American Society for Testing and Materials] Type 1 water [referred to as water]) to a final concentration of 1X LDS/DTT. For purity, western blot, and glycoprotein analysis, and for sample preparation for peptide mapping and N-terminal amino acid sequencing, Cry1Da2 protein test substance (~5 mg) was solubilized in 1X LDS/DTT buffer to a final concentration of 1.0 mg/ml.

The samples were heated at 90-100 °C for 5 minutes and stored frozen (-20 °C freezer unit). Samples were reheated and diluted (as applicable) prior to SDS-PAGE analysis.

SDS-PAGE Analysis

The prepared protein samples were re-heated if applicable and analyzed using 4-12% Bis-Tris gels. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also 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 SDS 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 for concentration and purity analysis, western blot analysis, protein glycosylation analysis, or sample preparation for N-terminal amino acid sequencing and peptide mapping.

For protein concentration analysis: Five individually weighed samples of the Cry1Da2 protein were solubilized as described in Method Section A. The Cry1Da2 reference standard was used to prepare a standard curve at concentrations of 75 ng/µl, 62.5 ng/µl, 50 ng/µl, 37.5 ng/µl, and 25 ng/µl in 1X LDS/DTT. The standard curve, five Cry1Da2 diluted samples, and molecular weight markers were loaded (20 µl per lane) onto three separate gels and electrophoresed. Following electrophoresis, the gels were washed three times with water for 5 minutes each and stained with GelCode Blue stain reagent for 60 minutes. Following staining, the gels were de-stained with water four times for a minimum of 5 minutes each until the gel background was clear. Proteins

were stained as blue-colored bands on the gels. The gel image was captured electronically using a ChemiDoc MP imaging system. Densitometry analysis of the gels was conducted using Image Lab Software to interpolate the Cry1Da2 protein samples from the standard curve. The average of the five Cry1Da2 protein sample results on the three gels was used to determine the protein concentration of the Cry1Da2 test substance in the powder. The acceptability criteria for the standard curve is an R^2 value > 0.9. The Cry1Da2 reference standard curve met the acceptability criteria.

For purity analysis: Following sample preparation, Cry1Da2 protein was diluted with 1X LDS/DTT to a concentration of 0.5 ng/µl. Cry1Da2 protein was loaded at 1 µg onto a gel in each of three lanes and electrophoresed. Following electrophoresis, the gel was washed, stained, and imaged as described in Methods Section B.1. With the image of the gel, densitometry analysis was conducted to evaluate the purity of the Cry1Da2 protein based on the relative intensity of the Cry1Da2 protein band within each lane using ChemiDoc MP imaging software. The relative intensity of the bands was averaged across the three lanes to determine purity. If the protein was a single band or the predominant band in the lane was greater than 95%, a purity of "> 95%" was assigned.

Western Blot Analysis

For western blot analysis, the 1.0 mg/ml sample in 1X LDS/DTT was diluted with 1X LDS/DTT to a concentration of 0.25 ng/µl. Cry1Da2 protein was loaded at 5 ng on to the gel. 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 phosphate-buffered saline containing polysorbate 20 (PBST) containing 5% weight/volume (w/v) non-fat dry milk for 50 minutes at ambient laboratory temperature. Before and after the blocking step, the membrane was washed with PBST three times for 5 minutes each to reduce the background. The blocked membrane was incubated for 45 minutes at ambient laboratory temperature with a Cry1Da2 polyclonal antibody (Dow; Lot # TG5072014) diluted 1:10,000 in PBST containing 1% w/v non-fat dry milk. Following primary antibody incubation, the membrane was washed three times in PBST for 5 minutes each. The membrane was incubated for 65 minutes at ambient laboratory temperature with a secondary antibody (anti-rabbit IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:10,000 in PBST containing 1% non-fat dry milk. The membrane was washed three times with PBST for at least 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 a ChemiDoc MP imaging system.

Peptide Mapping by Mass Spectrometry

For mass spectrometry sequencing analyses, the 1.0 mg/ml sample in 1X LDS/DTT was diluted with 1X LDS/DTT to a concentration of 0.2 µg/µl. Cry1Da2 protein was loaded at 4 µg on to a gel in each of three lanes. Following SDS-PAGE, the gels were washed three times with water for 5 minutes each and stained with GelCode Blue stain reagent for 60 minutes. Following staining, the gels were de-stained with water four times for a minimum of 5 minutes each until the gel

background was clear. Proteins were stained as blue-colored bands on the gels. The gel image was captured electronically using a ChemiDoc MP imaging system. Protein bands at the expected molecular weight of Cry1Da2 protein were excised from the gel and stored frozen (< -5 °C). 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 an ACQUITY UPLC (Waters Corporation) fitted with a Cortecs UPLC C18 1.6 µm column (2.1 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 MS data were processed using MS Data Converter (Beta 1.3) to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software version 2.7.0) and match peptides from the expected Cry1Da2 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 12.11.0.

N-Terminal Amino Acid Sequencing Analysis

For N-terminal amino acid sequence analyses, the 1.0 mg/ml sample in 1X LDS/DTT was diluted with 1X LDS/DTT to a concentration of 0.2 mg/ml. Cry1Da2 protein was loaded at 4 µg on to a gel in each of three lanes. 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 PVDF membrane was briefly wetted in 100% methanol, 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 45 minutes. Following protein transfer, the membrane was washed with water three times for 5 minutes each wash. The membrane was then stained with GelCode Blue stain reagent for 5 minutes and then destained with water to visualize protein bands at the expected molecular weight of Cry1Da2 protein. The bands were excised and stored frozen (< -5 °C) until analysis. Two bands 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 with applicable adjustments.

Glycoprotein Analysis

A Pierce Glycoprotein Staining Kit was used to determine whether the Cry1Da2 protein was glycosylated. For glycosylation staining, the 1.0 mg/ml sample in 1X LDS/DTT was diluted with 1X LDS/DTT to a concentration of 0.05 µg/µl. Cry1Da2 protein was loaded at 1 µg on to the gel. The Cry1Da2 protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor), were run by SDS-PAGE.

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 three times with 3% acetic acid for 5 minutes and then rinsed three times in water for 5 minutes each. 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 (at least 5 minutes each) to visualize all protein bands. The image of the GelCode-stained gel was then captured electronically.

Bioactivity Bioassay

The biological activity of the Cry1Da2 protein was evaluated by conducting a 7-day bioassay using *Spodoptera frugiperda* (fall armyworm; Lepidoptera: Noctuidae), a species sensitive to the Cry1Da2 protein. Eggs were obtained from [redacted] and their identity was recorded by study personnel.

The *S. frugiperda* bioassay utilized a generalized randomized block design containing four blocks. Each block consisted of a 12-well bioassay plate and contained five replicates from each of the following treatments for a target of 20 individuals per treatment:

- Treatment 1: Bioassay Control Diet (containing a dosing solution of ultrapure water)
- Treatment 2: Test Diet (targeting 50 ng Cry1Da2 protein per mg diet wet weight)

A Cry1Da2 protein stock solution was prepared by solubilizing ~ 5 mg of test substance in ultrapure water to a concentration of 1.0 mg/ml. To prepare the test dosing solution, the stock solution was diluted in ultrapure water to a concentration of 66.7 ng/µl. The bioassay control dosing solution consisted of ultrapure water. The Cry1Da2 protein stock solutions and dosing solutions were prepared on Day 0 and Day 4 of the *S. frugiperda* bioassay and maintained chilled on wet ice until use. The carrier for the *S. frugiperda* bioassay consisted of Stonefly Heliothis diet. Each dosing solution was combined with carrier at a 3:1 ratio (i.e., 3 ml of dosing solution to 1 g carrier) to generate Treatments 1 and 2.

S. frugiperda eggs were incubated in an environmental chamber until the eggs hatched. Neonates were used in the bioassay within 24 hours of hatching. On Day 0 of the bioassay, approximately 300 µl (i.e., 1 g of wet diet equated to 1 ml of wet diet) of freshly prepared diets were dispensed into individual wells of the bioassay plates. One *S. frugiperda* neonate was placed in each well containing diet, each bioassay plate was sealed with heat-sealing film and a small hole was poked over each well to allow for ventilation. The bioassay was conducted in an environmental chamber set at 25 °C, 65% relative humidity, and continuous dark for 7 days. On Day 4, new bioassay plates were prepared with fresh diet as described for Day 0, with the exception that on Day 4, 600 µl was dispensed per well. Living *S. frugiperda* larvae were transferred to the new plates, missing

or dead larvae were recorded, and the freshly prepared plates were placed in the environmental chamber. After 7 days, the bioassay was complete and mortality was assessed.

The bioassay acceptability criterion indicated the bioassay may be repeated if the combined number of dead and missing organisms exceeds 20% for the bioassay control diet (Treatment 1) group. The bioassay met the acceptability criteria.

APPENDIX F. METHODS FOR CHARACTERIZATION OF DGT28 EPSPS PROTEIN

Test Materials

Maize derived DGT28-EPSPS Protein: DGT-28 EPSPS protein was isolated from DAS1131 maize leaf tissue. The tissue samples were collected at the R1 growth (the stage when silks become visible; Abendroth *et al.*, 2011) from plants grown at a field location in Johnston, IA, USA. The tissue was lyophilized, homogenized and stored at ≤ -50 °C.

The DGT-28 EPSPS protein was extracted from lyophilized maize tissue by homogenization in a pre-chilled Waring blender vessel using 50 mM Tris, pH 8, extraction buffer with EDTA-free Complete Protease Inhibitors. The sample extract was then filtered through cheesecloth, clarified by centrifugation, and fractionated using ammonium sulfate (AS) precipitation. Beginning at 0% AS saturation and using an online calculator by EnCor Biotechnology Inc. (EnCor Biotechnology, 2020), AS was slowly added to the sample extract while stirring until 30% AS saturation was reached. The sample was centrifuged and the AS process was repeated with the supernatant, this time beginning at 30% AS saturation and progressing to 60% AS saturation. The sample was centrifuged again, the supernatant was discarded, and the fractionated pellets were stored frozen (-80 °C freezer unit).

To prepare the sample for immunoaffinity purification, the pellets were solubilized and buffer-exchanged in chilled 50 mM Tris, pH 8, using Econo-Pac 10DG desalting columns from BioRad. Following buffer exchange, the sample was further purified by immunoaffinity chromatography. The immunoaffinity columns were prepared by coupling two DGT-28 EPSPS monoclonal antibodies (SW4-8F11 and SW4-18F5; Pioneer Hi-Bred International, Inc.) to AminoLink Plus Coupling Gel. The samples were eluted off the column using IgG Elution buffer with 1% CHAPS. Elutions 2-8 from the immunoaffinity purification were collected separately and immediately neutralized with 0.1 column volume of 1M Tris buffer, pH 8. The elutions were combined and concentrated using a centrifugal concentrator (30K Vivaspin Turbo 4; Sartorius), buffer-exchanged with 50 mM Tris buffer, pH 8, to a volume of 500-900 µl, and stored frozen (-80 °C freezer unit).

The partially purified maize-derived DGT-28 EPSPS protein samples were removed from the freezer and thawed on wet ice. The thawed samples were combined and concentrated into one sample using a centrifugal concentrator to a volume of 100 µl. An equal volume of 2X LDS/DTT (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 in the centrifugal 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 (-20 °C freezer unit).

Microbially Derived DGT-28 EPSPS Protein: DGT-28 EPSPS protein was produced at Pioneer Hi-Bred International, Inc. using an *E. coli* protein expression system as a fusion protein with an N-terminal His tag. The tagged protein was purified using Ni-NTA affinity chromatography. The protein was buffer-exchanged using tangential flow filtration to change the buffer to 25 mM glycine-KOH, 1 mM KH_2PO_4, 0.5 mM EDTA, 25 mM Trehalose, 0.5 mM TCEP, pH 9.5. After lyophilization and mixing, a lot number (PCF-0054) was assigned.

<u>SDS-PAGE Analysis</u>

Maize derived DGT28-EPSPS Protein: Sample was diluted as applicable, re-heated for 5 minutes at 90-100 °C, and then loaded into 4-20% Tris-Glycine or 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 range of the predicted molecular weight. For SDS-PAGE and western blot analyses, the DGT-28 EPSPS protein reference substance was also re-heated for 5 minutes at 90-100 °C and loaded to approximately the same concentration as the maize-derived protein. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X Tris-Glycine or 1X MES running buffer at a constant 200 volts (V) for 35-45 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 DGT28-EPSPS Protein: Protein was solubilized to a target concentration of 1 mg/ml based on amino acid analysis in 1X LDS/DTT sample buffer (25% 4X NuPAGE LDS Sample Buffer, 10% 10X NuPAGE Sample Reducing Agent containing DTT, and 65% ASTM [American Society for Testing and Materials] Type I water [referred to as water]) and heated at 90-100 °C for 5 minutes. The sample was stored frozen (-20 °C freezer unit) until SDS-PAGE analysis. The aliquots of solubilized DGT-28 EPSPS protein were diluted and reheated as applicable prior to loading for SDS PAGE analysis. The prepared protein samples were analyzed using 4-12% Bis-Tris gels. For Coomassie staining and glycosylation staining, 1 µg of DGT-28 EPSPS protein was loaded. For western blot analysis, 5 ng of DGT-28 EPSPS protein was loaded. For mass spectrometry analyses, 4 µg of DGT-28 EPSPS protein was loaded. Pre-stained protein molecular weight markers (Precision Plus Protein Dual Xtra Standards) were also 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 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.

For Coomassie staining of maize and microbially derived samples, the gel was washed with water three times for at least 5 minutes each and stained with GelCode Blue Stain Reagent for 60-70 minutes. Following staining, the gel was de-stained with water four times for at least 5 minutes each or until the gel background was clear. Proteins were stained as blue-colored bands on the gels. The gel image was captured electronically using a ChemiDoc MP (Bio-Rad) imaging system.

<u>Western Blot Analysis</u>

Following SDS-PAGE, the resulting gel was assembled into a 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) containing 5% weight/volume (w/v) non-fat dry milk for approximately 60 minutes at ambient laboratory temperature.

Maize derived DGT28-EPSPS Protein: 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 DGT-28 EPSPS monoclonal antibody SW4-8F11 (Pioneer Hi-Bred International, Inc.) diluted 1:10,000 in PBST containing 1% (w/v) non-fat dry milk.

Microbially derived DGT28-EPSPS Protein: Before the blocking step, the membrane containing was washed with PBST three times for 1 minutes each to reduce the background. The blocked membrane was incubated in an DGT-28 EPSPS monoclonal antibody diluted 1:10,000 (maize-derived) or 1:10,000 (microbially derived) 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 four times for 5 minutes each. The membrane was incubated for 60 minutes at ambient laboratory temperature with a secondary antibody (anti-mouse IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:20,000 in PBST containing 1% non-fat dry milk. The membrane was washed three-four times with PBST for at least 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 a ChemiDoc MP imaging system.

Peptide Mapping by Mass Spectrometry

Following SDS-PAGE, Coomassie staining, and imaging of the gels, the DGT-28 EPSPS protein bands were excised from the gel and and stored frozen at ≤ -5 °C.

Maize derived DGT28-EPSPS Protein: Gel slices were reduced with DTT, alkylated with iodoacetamide, and then subsequently digested chymotrypsin. A digested sample was 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 MS data were processed using MS Data Converter (Beta 1.3) to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software version 2.7.0) and match peptides from the expected DGT-28 EPSPS 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 2 maximum missed cleavages. The Mascot-generated peptide ion score threshold was > 13 which indicates identity or extensive homology ($p < 0.05$).

Microbially derived DGT28-EPSPS Protein: Gel slices were reduced with DTT, alkylated with iodoacetamide, and then subsequently digested with trypsin or chymotrypsin. The digested samples were separated on an ACQUITY UPLC (Waters Corporation) fitted with a Cortecs UPLC C18 1.6 µm column (2.1 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 MS data were processed using MS Data Converter (Beta 1.3) to produce a peak list. The peak list was used to perform an MS/MS ion search (Mascot Software version 2.7.0) and match peptides from the expected DGT-28 EPSPS 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 12.11.0.

N-Terminal Amino Acid Sequencing Analysis

Maize derived DGT28-EPSPS Protein: Following SDS-PAGE as described in Methods Section B, 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 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 twice with water for 5 minutes each wash, stained with GelCode Blue stain reagent for 5 minutes, and then de-stained with water to visualize the DGT-28 EPSPS protein. Two bands containing the maize-derived DGT-28 EPSPS protein were excised and stored frozen at ≤ -5 °C. The bands 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. The N-terminal sequence was manually identified.

Microbially derived DGT28-EPSPS Protein: Following SDS-PAGE, Coomassie staining, and gel imaging using the methods as described above, the resulting gel was incubated in cathode buffer cathode buffer (60 mM Tris, 40 mM CAPS, 0.075% SDS, pH 9.6) for 10-20 minutes. An Immobilon-P PVDF membrane was briefly wetted in 100% methanol, 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. The membrane was then stained with GelCode Blue stain reagent for 3 minutes and then destained with water to visualize protein bands at the expected molecular weight of DGT-28 EPSPS protein. The bands were excised and stored frozen (< -5 °C) until analysis. Two bands were analyzed as a single sample using a Shimadzu PPSQ-51A sequencer. Fifteen 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 with applicable adjustments.

Glycoprotein Analysis

A Pierce Glycoprotein Staining Kit was used to determine whether the maize derived DGT28-EPSPS Protein or the microbially derived DGT28-EPSPS Protein was glycosylated. The DGT-28 EPSPS protein, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor), were run by SDS-PAGE.

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 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 three times with 3% acetic acid for at least 5 minutes and then rinsed once 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 approximately 60 minutes followed by three washes with water (at least 5 minutes each) to visualize all protein bands. The image of the GelCode-stained gel was then captured electronically.

<u>Enzymatic Assay of the Microbially derived DGT28-EPSPS Protein:</u>

DGT-28 EPSPS is an EPSP synthase (or EPSPS) enzyme in the shikimate pathway that catalyzes the reaction of shikimate-3-phosphate (S3P) with phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate 3-phosphate and inorganic phosphate. DGT-28 EPSPS activity was determined by measuring the quantity of free phosphate released as a product by the reaction (Forlani *et al.*, 1994; Lanzetta *et al.*, 1979).

Microbially derived DGT28-EPSPS Protein: A 5.3-mg lyophilized DGT-28 EPSPS protein sample was solubilized under chilled conditions using 50 mM Tris, pH 8.0, to a target concentration of 1 mg/ml. The solution was vortexed for 10-30 seconds, allowed to rest for 5-10 minutes, and then vortexed for an additional 10-30 seconds. The solubilized protein was diluted using 1X assay buffer (50 mM HEPES, 100 mM KCl and 2 mM DTT, pH 7.5).

A standard curve of 90, 60, 30, 15, 5, and 0 µM was prepared by diluting a stock solution of KH_2PO_4 (100 mM KH_2PO_4 and 0.05 N HCl) in 1X assay buffer. The standard curve was plated in triplicate wells (50 µl per well). The solubilized DGT-28 EPSPS protein and a substrate blank included to account for background caused by the reactants were plated in duplicate wells (50 µl per well). Each sample well included 2 µl of diluted DGT-28 EPSPS protein sample in 48 µl of reaction buffer (20X assay buffer with DTT, S3P, PEP, and ultrapure water) and each blank well included 2 µl of 1X assay buffer in 48 µl of reaction buffer. The reaction was performed at final concentrations of 16 nM DGT-28 EPSPS, 50 mM HEPES, 100 mM KCl, 2 mM DTT, 1 mM S3P, and 1 mM PEP. After 15 minutes, 235 µl of a malachite green solution (comprised of 3 ml 0.045% malachite green in water to 1 ml of 4.2 % ammonium molybdate in 4N HCl) was added to each well (standard, blanks, and samples) and color was allowed to develop for 1-5 minutes. Absorbance was read at 660 nm using a SPECTRAmax M2 ROM plate reader.

SoftMax Pro GxP was used to derive the standard curve which used a quadratic fit to interpolate the average sample values (µM) of free phosphate released. The interpolated sample concentration values obtained from SoftMax Pro GxP software were used to calculate enzyme activity as follows:

$$\text{Enzyme activity } (PO_4 \text{ produced}) \text{ per minute} = \frac{\text{Sample value } (\mu M) * \text{Reaction volume(L)}}{}$$

(µmol/min) Reaction time (min)

The amount of DGT-28 EPSPS protein (mg) in the reaction was calculated as follows:

$$\text{DGT-28 EPSPS (mg)} = \text{Protein Concentration in Reaction (M)} * \text{Molecular Weight} * \text{Reaction Volume (L)}$$

Enzyme activity (µmol/min) and mg of DGT-28 protein were used to calculate the specific activity of the DGT-28 EPSPS enzyme as follows.

$$\text{Specific activity } (\mu\text{mol/min/mg protein}) = \frac{\text{Enzyme activity } (\mu\text{mol/min})}{\text{DGT-28 EPSPS (mg)}}$$

Thermolability Analysis

DGT-28 EPSPS is a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway that catalyzes the reaction of shikimate-3-phosphate (S3P) with phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate 3-phosphate and inorganic phosphate. DGT-28 EPSPS activity was determined by measuring the quantity of free phosphate released as a product by the reaction of S3P and PEP (Forlani *et al.*, 1994; Lanzetta *et al.*, 1979).

A 5.3-mg sample of DGT-28 EPSPS protein (PCF-0054) was solubilized in 2.44 ml of ultrapure (ASTM [American Society for Testing and Materials] Type I) water to a target concentration of 1 mg/ml. The sample was vortexed for 10-30 seconds and then allowed to rest for 5-10 minutes. Aliquots (400 µl) were dispensed into 1.5-ml Eppendorf Protein LoBind tubes for heat treatment for 30-35 minutes using a heat block set to obtain temperatures (± 5 °C) of 25 °C, 37 °C, 50 °C, and 75 °C. One vial was left chilled (2-8 °C or on wet ice) as an unheated control treatment. Following heat treatment, samples were prepared for enzymatic activity analysis. Samples were maintained chilled when not under heat treatment.

Prior to enzymatic activity analysis, the heat-treated samples and unheated control sample were diluted using 1X assay buffer (50 mM HEPES, 100 mM KCl, and 2 mM DTT, pH 7.5) to a final concentration of 1 µM.

A standard curve of 90 µM, 60 µM, 30 µM, 15 µM, 5 µM, and 0 µM was prepared by diluting a stock solution of KH_2PO_4 (100 mM KH_2PO_4 and 0.05 N HCl) in 1X assay buffer. The standard curve was plated in triplicate wells (50 µl per well). The solubilized DGT-28 EPSPS protein and a substrate blank included to account for background caused by the reactants were plated in duplicate wells (50 µl per well). Each sample well included 2 µl of diluted DGT-28 EPSPS protein sample in 48 µl of reaction buffer (20X assay buffer with DTT, S3P, PEP, and ultrapure water) and each blank well included 2 µl of 1X assay buffer in 48 µl of reaction buffer. The reaction was performed at final concentrations of 40 nM DGT-28 EPSPS, 50 mM HEPES, 100 mM KCl, 2 mM DTT, 1 mM S3P, and 1 mM PEP. After 15 minutes, 235 µl of a malachite green solution (comprised of 7.5 ml of 0.045% malachite green in water to 2.5 ml of 4.2% ammonium molybdate in 4N HCl) was added to each well (standard, blanks, and samples) and color was allowed to

develop for 1-5 minutes. Absorbance was read at 660 nm using a SPECTRAmax M2 ROM plate reader.

SoftMax Pro GxP was used to derive the standard curve which used a quadratic fit to interpolate the average sample values (µM) of free phosphate released. The interpolated sample concentration values obtained from SoftMax Pro GxP software were used to calculate enzyme activity as follows:

$$\text{Enzyme activity } (PO_4 \text{ produced}) \text{ per minute } (\mu\text{mol/min}) = \frac{\text{Average interpolated sample value } (\mu\text{M}) * \text{Reaction volume (L)}}{\text{Total length of reaction time (min)}}$$

Digestibility in Simulated Gastric Fluid (SGF)

Test and control solutions were prepared as follows:

- The gastric control solution was prepared fresh on the day of use and was comprised of 0.2% weight per volume (w/v) NaCl in 0.7% volume per volume (v/v) HCl with a pH of ~1.2.
- The pepsin digestion solution, referred to as simulated gastric fluid (SGF), was prepared fresh on the day of use by dissolving pepsin (Sigma-Aldrich) into gastric control solution. The SGF was prepared so that the pepsin to protein ratio of the final digestion mixture was 10 units of pepsin per µg of test substance.
- The DGT-28 EPSPS protein stock solution was prepared fresh on the day of use by solubilizing a 5.3 mg sub-sample of DGT-28 EPSPS protein powder (PCF-0054) in 0.487 ml of ASTM [American Society for Testing and Materials] Type I ultrapure water to a target DGT-28 EPSPS protein concentration of 5 mg/ml.
- To prepare the stock solutions for each of the control proteins (BSA and β-lactoglobulin), a sub-sample of 5.0 mg powder was weighed into an individual tube and solubilized by adding 1 ml of water (for a final protein concentration of 5 mg/ml).
- The final concentration of the protein and pepsin in the SGF reaction mixture was 0.25 mg/ml DGT-28 EPSPS protein and 2500 units/ml pepsin.

SGF solution (1,900 µl) was dispensed into a 7-ml glass vial and placed in a 37 °C water bath for 2-5 minutes prior to the addition of 100 µl of DGT-28 EPSPS protein stock solution at Time 0. The digestion reaction mixture was mixed constantly using a stir bar and a submersible magnetic stirrer.

A 120-µl sub-sample of the DGT-28 EPSPS protein digestion reaction mixture was removed from the vial at the following analytical time points (± 10 seconds): 0.5, 1, 2, 5, 10, 20, and 60 minutes. The sub-samples were inactivated by adding them to pre-labeled tubes containing 139 µl of a pre-mixed sample stop solution (consisting of 48 µl of 200 mM sodium carbonate, 65 µl NuPAGE

4X LDS sample buffer, and 26 µl NuPAGE 10X sample reducing agent) and heating to 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

To prepare control digestion samples at 1 and 60 minutes, a 114-µl sample of the appropriate digestion solution (see table) was pre-warmed in a 37 °C water bath for 2-5 minutes prior to adding 6 µl of the DGT-28 EPSPS protein stock solution, control protein stock solution, or water. The tubes were incubated in the water bath for the allotted time and then inactivated by mixing with 139 µl of the pre-mixed sample stop solution.

The Time 0 control reaction mixtures were prepared by first neutralizing 114 µl of the appropriate digestion solution with 139 µl of the pre-mixed sample stop solution, and then adding 6 µl of the DGT-28 EPSPS protein stock solution, control protein stock solution, or water to the appropriate tube and mixing.

Control digestion samples included in the SGF assay are provided in Table F.1. Following digestion and inactivation, all control reaction mixtures were heated at 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

Table F.1. Control Samples for Simulated Gastric Fluid (SGF) Digestibility Analysis

Protein	Digestion Solution	Digestion Time (min)		
		0	1	60
None (Water), (SGF Control)	SGF	X	--	X
BSA	SGF	X	X	X
β-Lactoglobulin	SGF	X	X	X
DGT-28 EPSPS	SGF	X	--	--
DGT-28 EPSPS	None (Water)	X	--	X
DGT-28 EPSPS	Gastric Control Solution (No Pepsin)	--	--	X

SDS-PAGE Analysis

The DGT-28 EPSPS protein digestion time-course samples and control samples were removed from frozen storage, heated at 90-100 °C for 6 minutes, and loaded (20 µl /well) into 4-12% Bis-Tris gels for SDS-PAGE analysis. To demonstrate the sensitivity of the SDS-PAGE gel and western blot analyses, an aliquot of the DGT-28 EPSPS protein in SGF (Time 0) sample was loaded into the gel at a 1:20 dilution (116 ng of DGT-28 EPSPS protein) for Coomassie staining, and at a 1:200 dilution (11.6 ng DGT-28 EPSPS protein) for the western blot. Pre-stained protein molecular weight markers (Precision Plus Dual Xtra Standards) were also 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 pre-cast gel electrophoresis system with 1X MES SDS running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes for use in Coomassie staining or western blot analyses. For protein staining, the gels were washed three times for 5 minutes each with water and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, the gels were destained with water four times for a minimum of 5 minutes

each until the gel background was clear. Proteins were stained as blue-colored bands on the gels. The gel image was captured electronically using a ChemiDoc MP (Bio-Rad) imaging system.

Western Blot Analysis

The DGT-28 EPSPS protein digestion time-course samples were also analyzed by western blot. Following SDS-PAGE, one of the resulting gels 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 phosphate-buffered saline containing polysorbate 20 (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 at least 1 minute each to reduce the background. The blocked membrane was incubated for 60 minutes at ambient laboratory temperature with an DGT-28 EPSPS polyclonal antibody DA418 (Dow AgroSciences LLC) diluted 1:100,000 in PBST containing 1% (w/v) non-fat dry milk. Following primary antibody incubation, the membrane was washed in PBST four times for 5 minutes each. The membrane was incubated for 60 minutes at ambient laboratory temperature with a secondary antibody (anti-rabbit IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:100,000 in PBST containing 1% (w/v) non-fat dry milk. The membrane was washed in PBST four times for at least 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 a ChemiDoc MP imaging system.

Digestibility in Simulated Intestinal Fluid (SIF)

Test and control solutions were prepared as follows:

- The pancreatin digestion solution, referred to as simulated intestinal fluid (SIF), was prepared fresh on the day of use by dissolving 26.4 mg of pancreatin (Sigma-Aldrich) into 5 ml of intestinal control solution (I-Con 1X buffer) to a final concentration of 0.5% weight per volume (w/v) pancreatin and 50 mM KH_2PO_4, pH 7.5.
- The DGT-28 EPSPS protein stock solution was prepared fresh on the day of use by solubilizing a 5.2-mg sub-sample of DGT-28 EPSPS protein powder (PCF-0054) in 0.478 ml of ASTM [American Society for Testing and Materials] Type I ultrapure water to a target protein concentration of 5.0 mg/ml.
- To prepare the stock solutions for each of the control proteins (BSA and β-lactoglobulin), a 5.0-mg sub-sample of powder was weighed into an individual tube for each control and solubilized by adding 1.0 ml of water (to a target protein concentration of 5.0 mg/ml).
- The final concentration of the protein and pancreatin in the SIF reaction mixture was 0.25 mg/ml DGT-28 EPSPS protein and 0.5% (w/v) pancreatin.

SIF solution (1900 µl) was dispensed into a 7-ml glass vial and placed in a 37 °C water bath for 2-5 minutes prior to the addition of 100 µl of DGT-28 EPSPS protein test substance at Time 0. The digestion reaction mixture was mixed constantly using a stir bar and a submersible magnetic stirrer.

A 120-µl sub-sample of the DGT-28 EPSPS protein digestion reaction mixture was removed from the vial at the following analytical time points (± 10 seconds): 0.5, 1, 2, 5, 10, 20, 30, and 60 minutes. The sub-samples were inactivated by adding them to pre-labeled tubes containing 64 µl of pre-mixed sample solution (consisting of 46 µl NuPAGE 4X LDS Sample Buffer and 18 µl NuPAGE 10X Sample Reducing Agent) and heating to 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

To prepare control digestion samples at 1 and 60 minutes, a 114-µl sample of the appropriate digestion solution (see table) was pre-warmed in a 37 °C water bath for 2-5 minutes prior to adding 6 µl of the DGT-28 EPSPS protein test substance, control protein stock solution, or water. The tubes were incubated in the water bath for the allotted time and then inactivated by mixing with 64 µl of the pre-mixed sample solution.

The Time 0 control reaction mixtures were prepared by first neutralizing 114 µl of the appropriate digestion solution with 64 µl of the pre-mixed sample solution, and then adding 6 µl of the DGT-28 EPSPS protein test substance, protein stock solution, or water to the appropriate tube and mixing.

Following digestion and inactivation, all control reaction mixtures were heated at 90-100 °C for 5 minutes prior to frozen storage (-20 °C freezer unit).

Control digestion samples included in the SIF assay are provided in Table F.2.

Table F.2. Control Samples for Simulated Intestinal Fluid (SIF) Digestibility Analysis

Protein	Digestion Solution	Digestion Time (min)		
		0	1	60
None (Water), (SIF Control)	SIF	X	--	X
BSA	SIF	X	X	X
β-Lactoglobulin	SIF	X	X	X
DGT-28 EPSPS	SIF	X	--	--
DGT-28 EPSPS	None (Water)	X	--	X
DGT-28 EPSPS	Intestinal Control Solution (No Pancreatin)	--	--	X

SDS-PAGE Analysis

The DGT-28 EPSPS protein digestion time-course samples and control samples were removed from frozen storage, heated at 90-100 °C for 5 minutes, and loaded (10 µl/well) onto 4-12% Bis-Tris gels for SDS-PAGE analysis. Pre-stained protein molecular weight markers (Precision Plus Dual Xtra Standards) were also loaded onto each gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES SDS running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gels were removed from the gel cassettes for use in Coomassie staining or western blot analyses. For protein staining, the gels were washed three times for 5 minutes each with water and stained with GelCode Blue Stain Reagent for 60 minutes.

Following staining, the gels were destained with water four times for a minimum of 5 minutes each until the gel background was clear. Proteins were stained as blue-colored bands on the gels. The gel image was captured electronically using a ChemiDoc MP (Bio-Rad) imaging system.

Western Blot Analysis

The DGT-28 EPSPS protein digestion time-course samples were also analyzed by western blot. Following SDS-PAGE, one of the resulting gels 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 phosphate-buffered saline containing polysorbate 20 (PBST) containing 5% (w/v) non-fat dry milk for 50 minutes at ambient laboratory temperature. Before and after the blocking step, the membrane was washed with PBST three times for 5 minutes each to reduce the background. The blocked membrane was incubated for 45 minutes at ambient laboratory temperature with an DGT-28 EPSPS polyclonal antibody DA418 diluted 1:100,000 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. The membrane was incubated for 54 minutes at ambient laboratory temperature with a secondary antibody (anti-rabbit IgG, horseradish peroxidase conjugate; Promega Corporation) diluted 1:100,000 in PBST containing 1% (w/v) non-fat dry milk. The membrane was washed in PBST three times for at least 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 a ChemiDoc MP imaging system.

<u>Digestibility in sequential digestion in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)</u>

Test solutions were prepared as follows:

- A concentrated (i.e., 2X) pepsin digestion solution, referred to as simulated gastric fluid (SGF), was prepared fresh on the day of use by solubilizing pepsin (Sigma-Aldrich) in a previously prepared 2X gastric control solution. The final concentration of gastric control solution in SGF was 0.2% weight per volume (w/v) NaCl and 0.7% volume per volume (v/v) HCl ; pH ~1.2. The SGF was prepared so that the pepsin to protein ratio of the final digestion mixture was 10 units of pepsin per µg of test protein.
- A concentrated (i.e., 2.5X) pancreatin digestion solution, referred to as simulated intestinal fluid (SIF), was prepared fresh on the day of use by solubilizing pancreatin (Sigma-Aldrich) in 2.5X intestinal control solution (2.5X I-Con). The final concentration of intestinal control solution in SIF was 50 mM KH_2PO_4, with a pH of ~7.5. Pancreatin content in SIF was adjusted so that there was approximately 0.5% (w/v) pancreatin in the final digestion reaction mixture.
- The pre-mixed sample stop solutions used to inactivate samples were prepared fresh on the day of use. The solution for SGF reactions was prepared by mixing 1200 µl of 200 mM sodium carbonate, 1625 µl NuPAGE 4X LDS Sample Buffer, and 650 µl NuPAGE 10X DTT Sample Reducing Agent. The solution for SIF reactions was prepared by mixing 1150

µl NuPAGE 4X LDS Sample Buffer and 450 µl NuPAGE 10X DTT Sample Reducing Agent.

- The DGT-28 EPSPS protein stock solution was prepared fresh on the day of use by solubilizing a 5.2 mg sub-sample of DGT-28 EPSPS protein powder (PCF-0054) in 0.478 ml of ASTM [American Society for Testing and Materials] Type I ultrapure water to a target protein concentration of 5 mg/ml.

In Vitro Pepsin Digestion

DGT-28 EPSPS Protein in SGF 2 Minutes Sample for Sequential Digestion: An aliquot (1 ml) of the 2X SGF solution and 800 µl water were dispensed into a 7-ml glass vial and pre-warmed in the 37 °C water bath for 2-5 minutes prior to addition of 200 µl of the DGT-28 EPSPS protein test substance. The SGF digestion reaction mixture was incubated and mixed constantly using a stir bar and submersible stir plate for 2 minutes (± 10 seconds) after adding the DGT-28 EPSPS protein test substance. At the end of the time period, a 1.5-ml sample of the DGT-28 EPSPS SGF digestion reaction mixture was transferred to a separate vial and inactivated by neutralization with 0.3 ml of 0.5 N NaOH. This sample was used for the sequential SIF digestion.

DGT-28 EPSPS Protein in SGF 2 Minutes: A 120-µl control sample (DGT-28 EPSPS in SGF 2 minutes) was taken out from the SGF digestion reaction mixture at the end of 2 minutes (± 10 seconds) and inactivated by neutralization with 139 µl of pre-mixed SGF sample stop solution. The neutralized sample was heated for 5 minutes at 90-100 °C prior to frozen storage (-20 °C freezer unit).

DGT-28 EPSPS Protein in SGF Time 0: A control sample (DGT-28 EPSPS in SGF Time 0) was prepared by first inactivating 60 µl of 2X SGF and 49 µl water in 139 µl of pre-mixed SGF sample stop solution and then adding 12 µl of DGT-28 EPSPS protein test substance to the neutralized SGF. The neutralized sample was heated for 5 minutes at 90-100 °C prior to frozen storage (-20 °C freezer unit).

SGF-Only 2 Minutes Incubation: An SGF-only control sample without DGT-28 EPSPS protein test substance (SGF Control 1minute) was prepared by mixing 60 µl 2X SGF and 49 µl water in a tube and pre-warming at 37 °C for 2-5 minutes. Following the addition of 12 µl of water, the tube was incubated in a 37 °C water bath for 2 minutes (± 10 seconds). After incubation, the sample was inactivated by neutralization with 139 µl of pre-mixed SGF sample stop solution. The neutralized sample was heated for 5 minutes at 90-100 °C prior to frozen storage (-20 °C freezer unit).

Sequential Pancreatin Digestion

DGT-28 EPSPS Protein in SGF 2 Minutes, SIF 0.5-30 Minutes: For the sequential SIF digestion time course, a 1.2-ml sample of the neutralized DGT-28 EPSPS SGF digestion reaction mixture was dispensed into a 7-ml glass vial and placed in a 37 °C water bath for 2-5 minutes prior to addition of 800 µl 2.5X SIF solution. The SIF digestion reaction mixture was mixed constantly using a stir bar and a submersible stir plate.

A 120-µl sub-sample of the SIF digestion reaction mixture was removed from the vial at each of the following analytical time points (± 10 seconds): 0.5, 1, 2, 5, 10, 20 and 30 minutes. Each sub-sample was neutralized by adding it to a pre-labeled tube containing 64 µl of pre-mixed SIF sample stop solution. The neutralized samples were inactivated by heating at 90-100 °C for 5 minutes.

DGT-28 EPSPS Protein in SGF 2 Minutes, SIF Time 0: A SIF control sample (DGT-28 EPSPS 2 minutes SGF Time 0 SIF) was prepared by mixing 48 µl 2.5X SIF with 64 µl of pre-mixed SIF sample stop solution and then heating for 5 minutes at 90-100 °C. A sub-sample (72 µl) of the neutralized DGT-28 EPSPS SGF digestion reaction mixture was added to the heat-inactivated SIF control sample and then heated again for 5 minutes at 90-100 °C.

After neutralization and heating, all SIF reaction samples were stored frozen (-20 °C freezer unit).

SDS-PAGE Analysis

The digestion samples were removed from frozen storage, heated at 90-100 °C for 5 minutes, and loaded (10 µl/well) into a 4-12% Bis-Tris gel for SDS-PAGE analysis. Pre-stained protein molecular weight markers (Precision Plus Dual Xtra Standards) were also loaded into the gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using a pre-cast gel electrophoresis system with 1X MES running buffer at a constant 200 volts (V) for 35 minutes.

Upon completion of electrophoresis, the gel was removed from the gel cassette and washed three times for 5 minutes each with water and stained with GelCode Blue Stain Reagent for 60 minutes. Following staining, the gel was destained with water four times for a minimum of 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.

APPENDIX G. METHODS FOR TRAIT EXPRESSION ANALYSES

Field Trial Experimental Design

A multi-site field trial was conducted during the 2020 growing season at six sites in commercial maize-growing regions of the United States (one site in Illinois, Nebraska, and Texas; two sites in Iowa) 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.

Sample Collection

The following tissue samples were collected: Leaf (V6, V9, R1, and R4 growth stages), root (V9, R1, and R4 growth stages), pollen (R1 growth stage), stalk (R1 growth stage), forage (R4 growth stage), and grain (R6 growth stage). Growth stages are described in Table H.1. One sample per plot was collected for each tissue set. All samples were collected from impartially selected, healthy, representative plants to minimize potential bias.

Table G.1. 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 V6 growth stage leaf sample was obtained by pruning the youngest two leaves that emerged at least 8 in. (20 cm) in length from the plant. Each V9, R1, and R4 growth stage 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 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 3-4 in. (8-10 cm) or less in length and 1 cup (approximately 250 ml) of the root sections was collected into a pre-labeled, residue bag. At some sites, root tissue from more than one maize plant was pooled to achieve the sample volume.
- 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 stalk sample was obtained by pruning a section of stalk, directly above the primary ear node. The leaf sheath tissue was removed and the stalk section was cut in half length-wise. The half-sections were halved again, chopped into sections of 3-4 in. (8-10 cm) or less in length and collected into a pre-labeled, plastic-lined, cloth bag.
- 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-labelled, 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 selected ear. Each ear selected for sampling was a primary ear that had previously been self-pollinated. For each sample, a representative sub-sample of 50 ml was collected into an individual pre-labeled vial.

Sample Processing, Shipping, and Storage

Each sample was placed on dry ice within 10 minutes of collection in the field and transferred to frozen storage (-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, root and stalk tissues were sub-sampled to fill approximately ½ - ¾ of a pre-labeled 50-ml vial. The tissue was cut into small pieces less than 1 in. (2.5 cm) in length and the vial was placed onto dry ice once filled. All remaining root and stalk tissue were retained in the original vials/residue bags and stored frozen. Following lyophilization of the grain samples, 15 kernels from each grain sample were removed and placed in a corresponding vial and placed on dry ice. All remaining kernels were retained in the original 50-ml vial and moved back into frozen storage for retention.

Trait Confirmation

To confirm sample identity, event- and/or gene-specific polymerase chain reaction (PCR) analyses were performed for samples with unexpected ELISA results. If a given test sample was confirmed as not containing the event and/or gene of interest, the result was excluded from reporting.

Protein Concentration Determination

The concentrations of Cry1Da2 and DGT-28 EPSPS 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 and stalk. Samples analyzed for Cry1Da2 protein were extracted with 0.60 ml of chilled phosphate-buffered saline containing polysorbate 20 (PBST). Extracted samples were centrifuged, and then supernatants were removed and prepared for analysis. Samples analyzed for DGT-28 EPSPS protein were extracted with 0.60 ml of chilled H5 buffer with Triton X-100, which is 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, 0.3% polysorbate 20, and 1.0% Triton X-100. Extracted samples were centrifuged, and then supernatants were removed and prepared for analysis.

ELISA Methods

Cry1Da2 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 incubated in a plate pre-coated with a Cry1Da2-specific antibody. Following incubation, unbound substances were washed from the plate. A different Cry1Da2-specific antibody, conjugated to the enzyme horseradish peroxidase (HRP), was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound Cry1Da2-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.

DGT-28 EPSPS Protein ELISA Method: Prior to analysis, samples were diluted as applicable in H5 with Triton X-100 buffer. Standards (typically analyzed in triplicate wells) and diluted samples (typically analyzed in duplicate wells) were incubated in a plate pre-coated with a DGT-28 EPSPS-specific antibody. Following incubation, unbound substances were washed from the plate. A different DGT-28 EPSPS-specific antibody, conjugated to the enzyme HRP, was added to the plate and incubated. Unbound substances were washed from the plate. Detection of the bound DGT-28 EPSPS-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 Cry1Da2 and DGT-28 EPSPS 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:

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

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:

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

<u>Statistical Analysis</u>

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

APPENDIX H. METHODS FOR NUTRIENT COMPOSITION ANALYSIS

Field Trial Experimental Design

A multi-site field trial was conducted during the 2020 growing season at eight sites in commercial maize-growing regions of the United States (one site in Nebraska, Pennsylvania, and Texas, and two sites in Iowa and Illinois) and Canada (one site in Ontario). A randomized complete block design with four blocks was utilized at each site. Each block included DAS1131 maize, control maize, and four of the following reference maize lines: BK5883, P0843, XL5858, P0928, P0993, XL5939, MY09V40, XL5828, P1093, BK6076, 6046, P1151, XL6158, 33T56, MPS2H721, and BK6282 (collectively referred to as reference maize). An herbicide treatment of glyphosate was applied to DAS1131 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

Forage (R4 growth stage) and grain (R6 growth stage) samples were collected (one sample per plot) from self-pollinated plants in Rows 5 and/or 6 at the applicable growth stage. Growth stage descriptions are provided in Table H.1. All samples were collected from impartially selected, healthy, representative plants. Reference maize and control maize samples were collected prior to the collection of DAS1131 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.

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. The plants selected for forage sampling contained self-pollinated ears.

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

After collection, samples were placed in chilled storage (e.g., coolers with wet ice, artificial ice, or dry ice) and were transferred to a freezer (≤ -10 °C) or placed on dry ice within 3 hours of collection. Samples from each site were shipped frozen to [redacted] for analyses.

Nutrient Composition Analyses

The forage and grain samples were analyzed at [redacted]. 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, total dietary fiber (TDF), crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), 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 H.1. Methods for Compositional Analysis

Nutritional Analyte	Method
Moisture	The analytical procedure for moisture determination was based on a method published by AOAC International. Samples were analyzed 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	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	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	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. After extraction, the samples were oven dried and the crude fat content was determined gravimetrically.
Carbohydrates	The carbohydrate content, 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	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. After rinsing with reverse osmosis (RO) water, the remaining residue was dried and weighed to determine the crude fiber content.
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. After rinsing with RO water, the remaining residue was dried and weighed to determine the NDF content.
Acid Detergent Fiber	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 International. Samples were analyzed to determine the percentage of ADF by digesting with an acid detergent solution and washing with RO water. The remaining residue was dried and weighed to determine the ADF content.

Table H.1. Methods for Compositional Analysis (continued)

Nutritional Analyte	Method
Total Dietary Fiber	The analytical procedure for the determination of total dietary fiber in grain was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology), 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 was precipitated with ethanol. The precipitate (residue) was filtered, washed, dried, and then 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 procedures for the determination of minerals were based on methods published by AOAC International and CEM Corporation. The maize forage minerals determined were calcium and phosphorus. Additional grain minerals determined were copper, iron, magnesium, manganese, potassium, sodium, and zinc. The samples were digested in a microwave-based digestion system and the digestate was diluted using with deionized (DI) 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 were 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 were 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 microwave assisted ether extraction and base hydrolysis, to the fatty acid methyl ester (FAME) derivatives, which were analyzed by gas chromatography with flame ionization detection (GC/FID). Results were reported as percent total fatty acids but presented in the raw data as percent fresh weight.

Table H.1. Methods for Compositional Analysis (continued)

Nutritional Analyte	Method
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 DI water 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 set to measure absorbance at 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of nicotinic acid.
Pantothenic Acid (Vitamin B5)	The analytical procedure for the determination of pantothenic acid (vitamin B5) was based on a method from 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 18-22 hours. After incubation, the bacterial growth was determined using a spectrophotometer set to measure absorbance at 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 18-22 hours. After incubation, the microbial growth was determined using a spectrophotometer set to measure absorbance at 600 nm. The absorbance readings were compared to a standard curve generated using known concentrations of pyridoxine hydrochloride.

Table H.1. Methods for Compositional Analysis (continued)

Nutritional Analyte	Method
Folic Acid (Vitamin B9)	The analytical procedure for determination of folic acid was based on a microbiological assay published by AACC. Samples were hydrolyzed and digested by protease and amylase enzymes to release the folates 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 in folic acid standards using a spectrophotometer set to measure absorbance 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 methods published by AOAC International and in the *Journal of AOAC International*. Samples were extracted using a 40:60 acetone:hexane with tert-butylhydroquinone (TBHQ) solution then analyzed by HPLC with UV detection.
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 from the sample extracts.
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 was derivatized with butylboronic acid and anlayzed by GC/FID, and extracted raffinose was 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 HPLC with UV detection.
p-Coumaric and Ferulic Acid	The analytical procedure for the determination of *p*-coumaric and ferulic acids was developed based on methods published in *Journal of Agricultural and Food Chemistry*, *The Journal of Chemical Ecology* and *Analytical Chemistry*. Samples were hydrolyzed with a 2N sodium hydroxide and extracted with ethyl ether after acidified with hydrochloric acid. The combined ethyl either layer was analyzed to determine the amounts of *p*-coumaric acid and ferulic acid by separating the total content of phenolic acids using reverse phase HPLC and UV detection.

Table H.1. Methods for Compositional Analysis (continued)

Nutritional Analyte	Method
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 DAS1131 maize and the control maize had < 50% of samples below the LLOQ, then an across-site mixed model analysis was conducted.
- If, either DAS1131 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 was 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 DAS1131 maize and the control maize had 100% of samples below the LLOQ, then statistical analyses was not performed.

Statistical Model for Across-Site Analysis

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

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

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

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 $\sim iid\ N(0, \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a. Subscript a represents the corresponding source of variation.

The residual maximum likelihood estimation procedure was utilized to generate estimates of variance components and entry means across sites. The estimated means are known as empirical best linear unbiased estimators (hereafter referred to as LS-Means). The statistical comparison was conducted by testing for a difference in LS-Means between DAS1131 maize and the control maize. The approximated degrees of freedom for the statistical test were derived using the Kenward-Roger method (Kenward and Roger, 1997). A significant difference was identified if a P-value was < 0.05.

For each analyte, goodness-of-fit of the model was assessed in terms of meeting distributional assumptions of normally, independently distributed errors with homogeneous variance. Deviations from assumptions were addressed using an appropriate transformation or allowing for 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 DAS1131 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 2019 in the United States, Canada, Chile, Brazil, and Argentina. The combined data represent 184 commercial maize lines and 185 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 DAS1131 maize contained individual values outside the tolerance interval, it was then compared to the respective literature range obtained from published literature (AFSI, 2021; 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 Pioneer's proprietary database.

If the range of DAS1131 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 biological 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.