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Supporting document 1

Safety assessment – Application A1281

Food derived from herbicide-tolerant and insect-protected corn line DP910521

Executive summary

Background

Application A1281 seeks approval for the sale and use of food derived from corn line DP910521 that has been genetically modified (GM) for tolerance to the herbicide glufosinate and protection from lepidopteran insect pests.

Protection against insect pests is conferred by the expression of the *cry1B.34* gene, which is a chimeric gene composed of DNA sequences from the following *Bacillus thuringiensis* genes: (1) a *cry1B*-class gene; (2) the *cry1Ca1* gene; and (3) the *cry9Db1* gene**.** The encoded Cry1B.34 protein causes damage to the midgut epithelium of certain lepidopteran insect larvae, resulting in insect death.

Tolerance to the herbicide glufosinate is achieved by expression of the maize-optimised *mo-pat* gene, derived from the bacterium *Streptomyces viridochromogenes*, encoding the enzyme phosphinothricin acetyltransferase (PAT). DP910521 also expresses the phosphomannose isomerase (PMI) protein from *Escherichia coli* strain K-12 as a selectable marker. The PAT and PMI proteins have been assessed previously by FSANZ, but this is the first time FSANZ has assessed the Cry1B.34 protein.

This safety assessment addresses food safety and nutritional issues associated with the GM food. It therefore does not address:

- risks related to the environmental release of GM plants used in food production
- risks to animals that may consume feed derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of use

Corn has a long history of safe use in the food supply. Corn-derived products are routinely used in a large number and diverse range of foods e.g. cornflour, starch products, breakfast cereals and high fructose corn syrup.

Molecular characterisation

The genes encoding Cry1B.34 (*cry1B.34*), PAT (*mo-pat*), and PMI (*pmi*) were introduced into corn line DP910521 via a two-step transformation process. Molecular analyses indicate that a single copy of each of the linked *cry1B.34*, *mo-pat*, and *pmi* cassettes is present at a single insertion site in the DP910521 genome. There are no extraneous plasmid sequences or antibiotic resistance marker genes present in this line.

The introduced genetic elements were shown by molecular techniques and phenotypic analyses to be present within a single locus and stably inherited across multiple generations.

Characterisation and safety assessment of new substances

All three novel proteins (Cry1B.34, PAT and PMI) are expressed throughout DP910521. Expression levels are low in grain. Bioinformatic studies confirmed a lack of any significant amino acid sequence similarity between Cry1B.34 and known protein toxins or allergens. Laboratory studies demonstrated that the Cry1B.34 protein is susceptible to the action of digestive enzymes and would be thoroughly degraded before being absorbed during passage through the gastrointestinal tract. Cry1B.34 is also susceptible to heat inactivation at the high temperatures typically used in food processing.

Characterisation studies confirmed that the PAT and PMI proteins were identical to proteins previously assessed by FSANZ. Updated bioinformatic analyses for the PAT and PMI proteins were consistent with previous analyses showing that neither of these proteins shared any meaningful homology with any known allergens or toxins. Taken together, the evidence supports the conclusion that Cry1B.34, PAT and PMI are not toxic or allergenic to humans.

Herbicide metabolites

For PAT, the metabolic profiles resulting from the protein/herbicide interaction have been established through a significant history of use. There are no concerns that the spraying of corn line DP910521 with glufosinate would result in the production of metabolites that are not also produced in non-GM crops sprayed with the same herbicide and already used in the food supply.

Compositional analyses

Detailed compositional analyses were performed on DP910521. Statistically significant differences were found between grain from DP910521 and the non-GM control for 4 of the 66 analytes evaluated, however these differences were small and all within the range established for existing commercial non-GM corn cultivars. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in grain from DP910521 compared to non-GM corn cultivars available on the market.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant and insect-protected corn line DP910521. On the basis of the data provided in the present application and other available information, food derived from DP910521 is considered to be as safe for human consumption as food derived from non-GM corn cultivars.

Table of contents

EXECUTIVE SUMMARY **1**

1 INTRODUCTION **6**

2 HISTORY OF USE **6**

2.1 HOST ORGANISM 6
2.2 DONOR ORGANISMS 7

3 MOLECULAR CHARACTERISATION **8**

3.1 TRANSFORMATION METHOD 8
3.2 DETAILED DESCRIPTION OF INSERTED DNA 9
3.3 DEVELOPMENT OF THE CORN LINE FROM THE ORIGINAL TRANSFORMATION 12
3.4 CHARACTERISATION OF THE INSERTED DNA AND SITE(S) OF INSERTION 12
3.5 CONCLUSION 16

4 CHARACTERISATION AND SAFETY ASSESSMENT OF NOVEL SUBSTANCES **17**

4.1 CRY1B.34 17
4.2 PAT 22
4.3 PMI 23
4.4 PROTEIN EXPRESSION LEVELS IN DP910521 23
4.5 HERBICIDE METABOLITES 24

5 COMPOSITIONAL ANALYSIS **26**

5.1 KEY COMPONENTS 26
5.2 STUDY DESIGN 26
5.3 ANALYSES OF KEY COMPONENTS IN GRAIN 28

6 NUTRITIONAL IMPACT **30**

7 REFERENCES **31**

APPENDIX 1 ..... **35**

APPENDIX 2 ..... **36**

Index of Figures

	Title	Page
Figure 1	Map of plasmid PHP79620	9
Figure 2	Schematic of the inserted DNA in DP910521	10
Figure 3	Representation of the 3 genes from which the *cry1B.34 gene* is derived	18
Figure 4	Stages of corn growth	24
Figure 5	Mean expression levels of the Cry1B.34, PAT and PMI proteins in DP910521	25
Figure 6	Analytes measured in DP910521 grain samples	27
Figure 7	Visual summary of statistically significantly analyte differences in DP910521 compared to the conventional control	29

Index of Tables

	Title	Page
Table 1	Expression cassettes contained in the DP910521 insert	11
Table 2	DP910521 generations used for various analyses	12
Table 3	Segregation results in five generations of PHP910521	14
Table 4	Bioactivity of microbially-derived Cry1B.34 in a diet fed to insect larvae	19
Table 5	Bioactivity of heat-treated Cry1B.34 in a diet fed to insect larvae	21

List of Abbreviations

Abbreviation	Description
aa	amino acid(s)
ADF	acid detergent fibre
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
CAPS	*N*-cyclohexyl-3-aminopropanesulfonic acid
CCI	confidential commercial information
CI	confidence interval
COMPARE	COMprehensive Protein Allergen REsource
DNA	deoxyribonucleic acid
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
FASTA	fast alignment search tool – all
FSANZ	Food Standards Australia New Zealand
g	gram
GM	genetically modified
HDR	homology-directed repair
HFCS	high fructose corn syrup
kDa	kilodalton
LC-MS	liquid chromatography-mass spectrometry
LLOQ	lower limit of quantitation
mg	milligram
MT	million tons
NCBI	National Centre for Biotechnology Information
NDF	neutral detergent fibre
ng	nanogram
NGS	next generation sequencing
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PMI	phosphomannose isomerase
qPCR	quantitative polymerase chain reaction
RF	reading frame
SbS	Southern-by-sequencing
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SSI	site-specific integration
TDF	total dietary fibre
µg	microgram
USDA	United States Department of Agriculture
UTR	untranslated region

1 Introduction

FSANZ received an application from Corteva Agriscience Australia Proprietary Limited to vary Schedule 26 in the Australia New Zealand Food Standards Code. The variation is to include food from a new genetically modified (GM) corn line DP910521, with the OECD Unique Identifier DP-91Ø521-2. This corn line is tolerant to the herbicide glufosinate and protected against lepidopteran insect pests, including fall armyworm.

Protection against lepidopteran insect pests is conferred by the expression of the insecticidal *cry1B.34* gene encoding the insecticidal Cry1B.34 protein. The *cry1B.34* gene is composed of three DNA sequences derived from the following *Bacillus thuringiensis* genes: (1) a *cry1B*-class gene; (2) the *cry1Ca1* gene; and (3) the *cry9Db1* gene**.** FSANZ has assessed numerous previous applications for crops containing Cry proteins derived from *B. thuringiensis*, but this is the first time FSANZ has assessed the Cry1B.34 protein.

Tolerance to the herbicide glufosinate is achieved by expression of the maize-optimised *mo-pat* gene, derived from the bacterium *Streptomyces viridochromogenes*, encoding the enzyme phosphinothricin acetyltransferase (PAT). DP910521 also expresses the phosphomannose isomerase (PMI) protein from *Escherichia coli* strain K-12 as a selectable marker. The PAT and PMI proteins have been assessed previously by FSANZ.

If approved, food derived from DP910521 corn line may enter the Australian and New Zealand food supply as imported food products.

2 History of use

2.1 Host organism

The host organism is corn (*Zea mays*) which is also referred to as maize. The inbred corn line PH184C was used as the parental variety for the genetic modification described in this application.

Corn was one of the first plants to be cultivated by humans (Ranum et al. 2014) and is now the world's dominant cereal crop, with global production of 1,151 MT[1] in 2022/23, ahead of wheat (788 MT) and rice (513 MT) (USDA 2023). Due to its economic importance, corn has been the subject of extensive study[2].

The United States is the world's largest producer of corn, producing 349 MT in 2022/23 (USDA 2023). Of the corn grown in the United States, an estimated 92% is GM[3]. No GM corn is currently grown commercially in Australia or New Zealand.

Relatively small quantities of non-GM corn are grown in Australia and New Zealand. In 2021 these amounted to 0.306 and 0.209 MT respectively (FAOSTAT 2023). To supplement their limited local production of corn, Australia and New Zealand import both corn grain and processed corn products. For example, in 2021 the imported quantities of corn flour into Australia and New Zealand were 11,626 and 1,284 tonnes respectively, while imports of corn oil totalled 1,106 and 122 tonnes respectively (FAOSTAT 2023).

[1] million tons

[2] Refer to detailed reports published by the OECD (OECD 2002), the Grains Research and Development Corporation (GRDC 2017) and the Office of the Gene Technology Regulator (OGTR 2008).

[3] For more information please see USDA Economic Research Service: http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx

Corn has a long history of safe human consumption[4]. Food products derived from processing of corn kernels include corn flour, meal, oil, starch and sweeteners such as high fructose corn syrup (HFCS). In Australia and New Zealand, corn starch is used in dessert mixes and canned foods and HFCS is used in breakfast cereals, baking products, corn chips and extruded confectionary.

2.2 Donor organisms

2.2.1 Bacillus thuringiensis

The *cry1B.34* gene is derived from the bacterium *B. thuringiensis*. *B. thuringiensis* is a Gram-positive, endospore-forming bacterium, ubiquitous in soil and considered non-pathogenic to humans (Palma et al. 2014). *B. thuringiensis* expresses a number of insecticidal proteins, including the well-characterised Cry proteins, and various *B. thuringiensis* strains have a long history of use as biopesticides[5] (Nester et al. 2002; CERA 2011). Currently, there are approximately 44 biopesticide products based on *B. thuringiensis* registered in Australia (APVMA 2023) and 10 in New Zealand (ACVM 2023). The widespread use of *B. thuringiensis* in agriculture, along with its ubiquitous presence in soil and on vegetation, mean that this species has a long history of being safely consumed in food.

B. thuringiensis has been linked to human diarrheal illness due its close relationship with the species *Bacillus cereus*. Some *B. cereus* strains contain enterotoxin genes and are pathogenic to humans (Ehling-Schulz et al. 2019; Biggel et al. 2022). However, current evidence suggests the mechanism of action that underlies disease caused by certain *B. cereus* strains is not linked to strains of *B. thuringiensis* used in agriculture (Raymond and Federici 2017; Biggel et al. 2022).

2.2.2 Streptomyces viridochromogenes

The source of the *mo-pat* gene is the bacterium *S. viridochromogenes.* This Gram-positive, spore-forming species is found in soil and water and is not pathogenic to humans or animals. *S. viridochromogenes* itself does not have a history of use in food, but the *pat* gene has been used to confer glufosinate tolerance in food-producing crops for almost three decades (CERA-ILSI 2016).

2.2.3 Escherichia coli

The *pmi* gene is derived from the bacterial species *E. coli*, a Gram-negative bacterium which is ubiquitous in the environment. *E. coli* strain K-12 is a non-pathogenic strain with a long history of use for laboratory and commercial applications. Despite the pathogenicity of certain *E. coli* strains, such as the enterohaemorrhagic *E. coli* group (e.g. 0157:H7), there are no toxicity or health concerns associated with strain K-12.

2.2.4 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of DP910521. These genetic elements are non-coding sequences and are used to regulate the expression of *cry1B.34*, *mo-pat* and *pmi*.

[4] A large proportion of corn produced is also used as animal feed.
[5] Since 1938 in France and 1961 in the United States

3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA, including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Some details of the genetic elements and plasmids used in the construction of DP910521, as well as its breeding history, were provided in the application as Confidential Commercial Information (CCI). While the full details of CCI cannot be provided in this public report, FSANZ has had regard to this information in its assessment.

3.1 Transformation method

Two sequential transformation steps were used to construct corn line DP910521. Site-specific integration (SSI) was used to direct the inserted DNA to a specific target site within the corn genome (Gao et al. 2020). The transformation methodology is outlined in the flowchart in Appendix 1 and summarised below.

In the first transformation step, microprojectile co-bombardment with multiple plasmids was used to insert a “landing pad” sequence at a targeted site within the genome of corn line PH184C. The landing pad consisted of a *lox*P site, *ubi*ZM1 promoter region, FRT1 flippase recombinase target site, *npt*II gene, *pin*II terminator, and FRT87 flippase recombinase target site. It was flanked by two sequences identical to endogenous sequences at the intended insertion site in the corn genome, allowing the landing pad sequence to be inserted by homology-directed repair (HDR). The first transformation step also utilised helper plasmids which transiently expressed the corn-derived WUS2 and ODP2 proteins. WUS2 and ODP2 allowed for improved regeneration of corn plants following transformation (Lowe et al. 2016), but no elements from these helper plasmids were incorporated into the corn genome.

In the second transformation step, microprojectile co-bombardment with four plasmids was used to exchange a portion of the landing pad sequence for the *pmi, mo-pat* and *cry1B.34* gene cassettes from the trait plasmid PHP79620 (Figure 1). The three additional plasmids – PHP73572, PHP21875 and PHP5096 – that were co-transformed with the trait plasmid transiently expressed the WUS2, ODP2 and flippase recombinase proteins, respectively. The cassettes encoding these proteins were not integrated into the corn genome. The WUS2 and ODP2 proteins performed the same function as in the first transformation step. The flippase recombinase allowed the exchange of the *npt*II gene and *pin*II terminator, located between FRT1 and FRT87 sites in the landing pad, with the *pmi, mo-pat* and *cry1B.34* trait expression cassettes, located between homologous FRT1 and FRT87 sites in PHP79620 (recombination fragment region; Figure 1). This exchange retained the *ubi*ZM1 promoter region from the landing pad sequence, allowing it to facilitate expression of the *pmi* selectable marker gene.

Following both transformation steps regenerated plants were screened and those with the intended insertion and no unintended DNA sequences were selected for further development. Following evaluation of trait efficacy and agronomic performance, corn line DP910521 was selected.

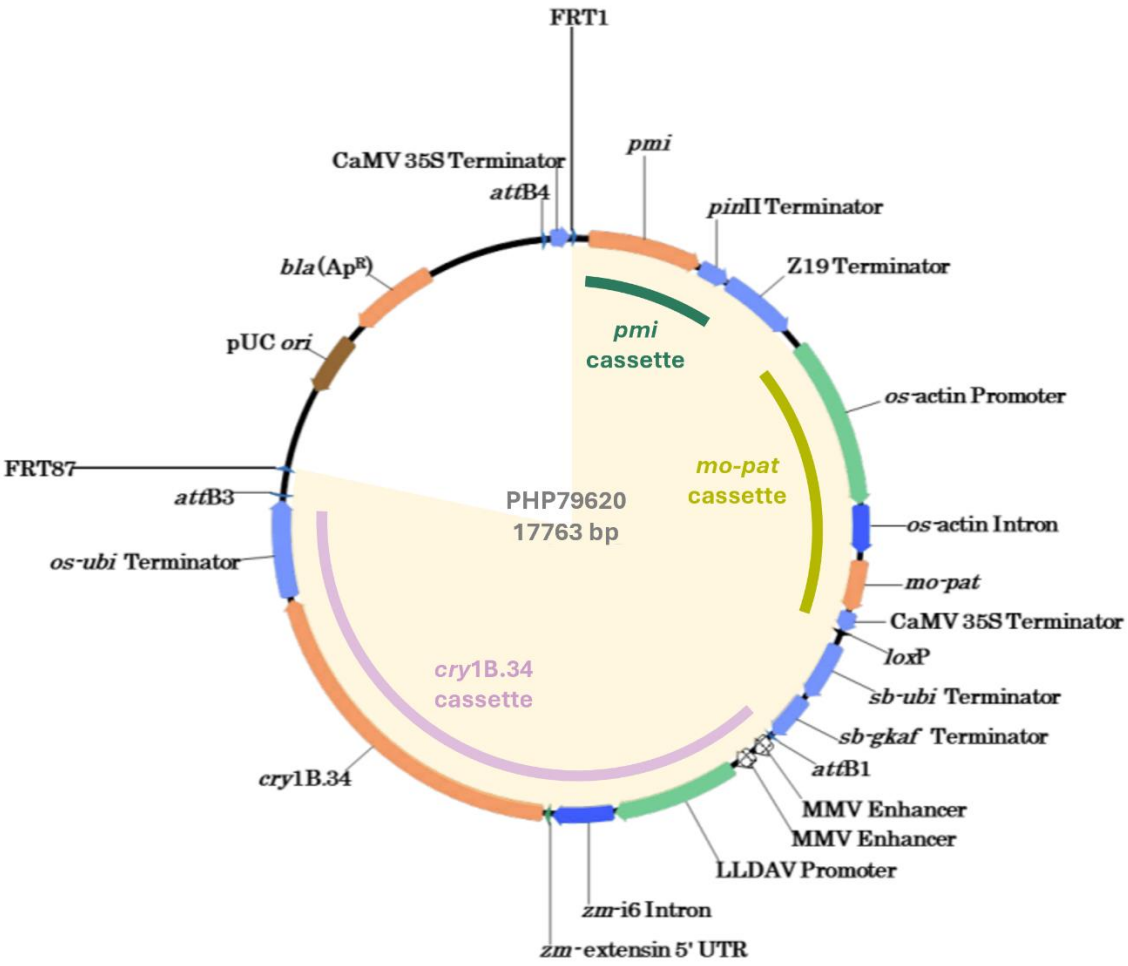

Figure 1. *Map of plasmid PHP79620. The region between the FRT1 and FRT87 sites (recombination fragment region) is shaded yellow and was inserted into the corn genome. This region contains the pmi, mo-pat and cry1B.34 expression cassettes.*

3.2 Detailed description of inserted DNA

Corn line DP910521 contains the recombination fragment region, which is found between the FRT1 and FRT87 sites in the PHP79620 plasmid (see Figure 1), as well as some residual DNA sequences derived from the landing pad plasmid (Figure 2). The *pmi*, *mo-pat* and *cry1B.34* expression cassettes are all derived from PHP79620, and are summarised in Table 1. The total size of the insertion is 16,269 bp. Additional detail, including factors transiently expressed to assist with transformation and intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in Appendix 2.

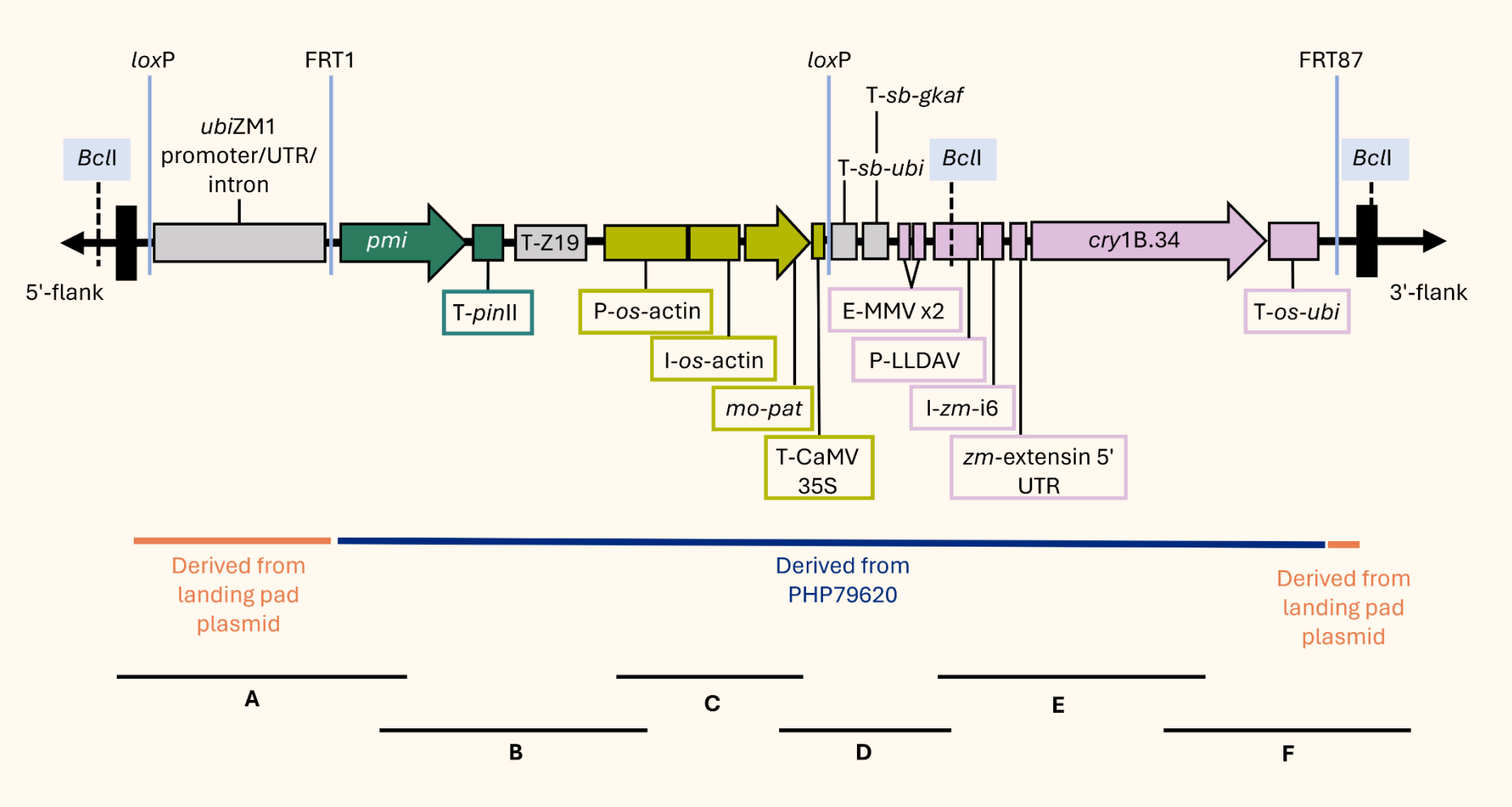

Figure 2. *Schematic of the inserted DNA in DP910521, derived from the landing pad plasmid (orange) and the trait plasmid PHP79620 (dark blue). The components of the pmi, mo-pat and cry1B.34 gene cassettes are coloured as per the plasmid diagram in Figure 1. The black rectangles at either end of the insert represent the endogenous corn genomic sequences used to insert the landing pad. The horizontal black lines labelled A-F represent the positions of the overlapping polymerase chain reaction fragments used for Sanger sequencing (see section 3.4.3). Bcl* I *restriction sites are shown and were used for Southern blot analysis (see section 3.4.4.1).*

Table 1: Expression cassettes contained in the DP910521 insert

	Promoter / Regulatory sequence(s)	Coding sequence	Terminator(s)	Function
***pmi* cassette**	Utilises the adjacent *ubi*ZM1 promoter from the landing pad sequence after incorporation into the genome	Phosphomannose isomerase (*pmi*) gene from *Escherichia coli*	Terminator from the proteinase inhibitor II (*pin*II) gene from *Solanum tuberosum* (potato) An additional terminator from the 19-kDa zein (Z19) gene from *Zea mays* (corn) is present between the *pmi* and *mo-pat* cassettes	Serves as a selectable marker during transformation
***mo-pat* cassette**	Promoter and intron region of the actin (*os-actin*) gene from *Oryza sativa* (rice)	Maize-optimised phosphinothricin acetyltransferase (*mo-pat*) gene from *Streptomyces viridochromogenes*	The 35S terminator region from Cauliflower mosaic virus (CaMV) Two additional terminators are present between the *mo-pat* and *cry1B.34* cassettes: the terminators from the ubiquitin (*sb-ubi*) and γ-kafirin (*sb-gkaf*) genes from *Sorghum bicolor* (sorghum)	Confers tolerance to glufosinate
***cry1B.34* cassette**	Two copies of the enhancer region from mirabilis mosaic virus (MMV) Promoter region from lamium leaf distortion-associated virus (LLDAV) Intron region from the translation initiation factor 6 (*zm*-i6) gene from *Z. mays* (corn) 5′ untranslated region (5′ UTR) from an extension gene from *Z. mays*	Chimeric gene comprised of sequences from a *cry1B*-class gene, the *cry1Ca1* gene, and the *cry9Db1* gene, all from *Bacillus thuringiensis*	Terminator from the ubiquitin (*os-ubi*) gene from *O. sativa*	Provides protection against susceptible lepidopteran insect pests

3.3 Development of the corn line from the original transformation

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of DP910521
- ensuring that the DP910521 event is incorporated into elite lines for commercialisation.

The generations analysed for the molecular characterisation and other analyses are listed in Table 2.

Table 2: DP910521 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Number of integration sites; insert organisation and the absence of plasmid backbone and other sequences	Sections 3.4.1, 3.4.2	T1	PH184C
Sanger sequencing	Section 3.4.3	BC1S2	PH184C
Genetic stability	Section 3.4.4.1	T1, BC1, BC1S1, BC1S2, BC1S3	PH184C
Mendelian inheritance; expression of phenotype over multiple generations	Section 3.4.4.2	F1, F2 BC1, BC1S1, BC1S3	N/A
Expression analysis of novel proteins	Section 4.4	F1	PH47K2xPH184C
Compositional analysis	Section 5	F1	PH47K2xPH184C

3.4 Characterisation of the inserted DNA and site(s) of insertion

A range of analyses were undertaken to characterise the genetic modification in DP910521. These analyses focused on the nature and stability of the inserted DNA and whether any unintended re-arrangements or products may have occurred as a consequence of the transformation procedure.

To characterise the number of integration sites, insert integrity and absence of extraneous sequences, the applicant made use of Southern-by-Sequencing (SbS) technology (Zastrow-Hayes et al. 2015; Brink et al. 2019). Sanger sequencing was used to determine the sequence of the inserted DNA and flanking sequences from the corn genome.

3.4.1 Number of integration site(s)

Leaf-derived genomic DNA from ten plants from the T1 generation of DP910521, along with DNA from a plant from the non-GM near-isogenic line PH184C as a control, was analysed by SbS. Additionally, positive control samples were generated using the PH184C genomic DNA spiked with the PHP79620 trait plasmid, the landing pad plasmid and each of the helper plasmids used during the two transformation steps. One copy of plasmid per copy of the corn genome was spiked.

Next generation sequencing (NGS) libraries were prepared using sheared genomic DNA consisting of an average fragment size of 400 bp. The probe set was designed to collectively target all sequences within all plasmids. The DNA was enriched twice by hybridisation and was sequenced using an Illumina platform. Sufficient sequence fragments were obtained to cover the genomes being analysed, with a 100x depth of coverage.

The sequencing reads obtained by SbS were compared to the intended insertion sequence, the plasmid sequences, and to the endogenous corn genome to identify unique junctions attributable to inserted DNA. The ten DP910521 plants analysed by SbS consisted of 6 GM plants containing the inserted DNA and 4 null segregant plants lacking the insert. SbS analysis of each of the 6 GM plants yielded sequencing reads that aligned to the intended insertion and identified two unique genome-insertion junctions. This result indicated that a single copy of the intended insertion was integrated into the genome of DP910521 (Figure 2).

The control contained sequencing reads that aligned with the intended insertion and plasmid sequences, but coverage above the background level was only obtained for endogenous sequences from corn that were present in the inserted DNA. No junctions between plasmid DNA and genomic DNA were identified in the PH184C control or in the 4 null segregant plants, confirming that the reads were only identifying endogenous sequences.

3.4.2 Absence of backbone and other sequences

The SbS analysis used a set of hybridisation probes covering the backbone sequences for all plasmids used in the development of DP910521. Alignment of NGS reads from the controls or DP910521 to all plasmid sequences confirmed there was no integration of backbone sequences into DP910521, including any antibiotic resistance genes.

3.4.3 Insert integrity and site of integration

The SbS analysis indicated that DP910521 contains a single copy of the intended insertion, with the expected organisation, and no unintended sequences or rearrangements.

Sanger sequencing was performed on six overlapping polymerase chain reaction (PCR) fragments covering the insert and flanking corn genomic sequences of DP910521 (see positions marked in Figure 2). The sequencing results confirmed that the insertion is 16,269 bp in length and comprised of the expected sequences derived from the PHP79620 trait and the landing pad plasmid.

3.4.4 Stability of the genetic changes in corn line DP910521

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification (as produced in the initial transformation events) over successive generations. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

3.4.4.1 Genetic stability

Southern blot analysis was used to show the genetic stability of the inserted *pmi, mo-pat and cry*1B.34 gene cassettes in DP910521. Leaf-derived genomic DNA from five generations of DP910521 (T1, BC1, BC1F1, BC1F2, BC1F3) was extracted, digested with the *Bcl* I restriction enzyme, and hybridised with labelled probes specific for the *pmi, mo-pat and cry*1B.34 gene cassettes. Genomic DNA from the non-GM corn line PH184C served as a negative control and PH184C DNA spiked with trait plasmid PHP79620 served as a positive control to confirm probe hybridisation.

Hybridisation of each probe to the digested genomic DNA from DP910521 showed equivalent bands of the expected sizes across all five generations. The consistency of these results confirmed that the inserted DNA is maintained stably in corn line DP910521.

3.4.4.2 Phenotypic stability

Expression of phenotype over several generations

The inheritance pattern of the inserted DNA was assessed in five generations of DP910521 (F1, F2, BC1, BC1S1, and BC1S3) using PCR assays with primers targeting the *pmi*, *mo-pat* and *cry1B.34* genes, as well as other genetic elements associated with the insertion site. For the F1, BC1 and BC1S3 generations, 100 plants from each generation were analysed; for the F2 generation, 252 seeds were analysed; and for the BC1S1 generation, 168 seeds were analysed. The F1, BC1, BC1S1, and BC1S3 generations were analysed using quantitative PCR (qPCR) to confirm the copy number of the inserted DNA, while the F2 generation was analysed by endpoint PCR to confirm the presence or absence of the DNA insert.

Plants were also examined phenotypically using a herbicide injury evaluation. Each plant was assessed visually for glufosinate-tolerance three to nine days after application of glufosinate spray. The absence of injury corresponded to a herbicide-tolerant (positive) phenotype.

Mendelian inheritance

A chi-square (χ^2) analysis was undertaken over several generations to confirm the segregation and stability of the insert in DP910521. Since the inserted DNA resides at a single locus within the DP910521 genome, the inserted DNA would be expected to be inherited according to Mendelian principles. The expected segregation ratios for each generation, based on Mendelian inheritance principles are shown in Table 3, along with the observed segregation results. No statistically significant ($p < 0.05$) deviation from the predicted segregation ratio was observed for the F1, BC1, BC1S1 or F2 generations. All plants from the BC1S3 generation were observed to be positive, as expected. These results aligned with the results from the phenotypic analysis for glufosinate tolerance, indicating that the PAT protein is stably expressed over multiple generations.

These data support the conclusion that the inserted DNA is present at a single locus in DP910521 and is inherited predictably according to Mendelian principles in subsequent generations.

Table 3: Segregation results in five generations of DP910521

Generation	Expected segregation ratio (positive:negative)	Observed number of plants/seeds			Statistical analysis	
		Positive	Negative	Total	χ^2	p-value
F1	1:1	46	53	99	0.49	0.4817
BC1	1:1	47	53	100	0.36	0.5485
BC1S3	Homozygous positive	100	0	100	-	-
BC1S1	3:1	130	35	165	1.26	0.2612
F2	3:1	175	68	243	1.15	0.2828

3.4.5 Reading frame analysis

A bioinformatic analysis of the DP910521 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel reading frames (RFs) had been created in DP910521 as a result of the DNA insertion, and whether any putative peptides encoded by the identified RFs have the potential for allergenicity or toxicity.

All sequences of ≥ 8 amino acids (aa) in length within the DP910521 insertion, or spanning

its boundaries with the flanking genomic DNA, were translated *in silico* from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames[6]. A total of 925 RFs ≥ 8 aa were identified and queried against allergen and toxin databases.

These analyses are theoretical only, as it is highly unlikely that any of the identified RFs or putative peptides would be expressed *in planta*.

3.4.5.1 Bioinformatic analysis for potential allergenicity

The amino acid sequences in the RFs identified above were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource (COMPARE[7]) database, from the Health and Environmental Science Institute. At the date of the search (January 2021), there were 2,348 sequences in the allergen database.

A FASTA search algorithm (v35.4.4) (Pearson and Lipman, 1988) was used to identify alignments between the query sequences and the COMPARE database, using a BLOSUM50 scoring matrix and an E-value threshold of 100. Only matches with a linear identity of greater than 35% over 80 amino acids were considered. In addition, a search for ≥ 8 contiguous aa matches to the allergens from the COMPARE database was performed using EMBOSS FUZZPRO.

Seven of the 925 putative peptides displayed > 35% identity with eleven known allergens over an 80 amino acid window. Further examination of each of these matches revealed that the chance of any production of viable allergenic peptides from these RFs would be remote, due to:

- a lack of available upstream promoters to drive transcription, and/or
- a lack of available start codons for translation initiation, and/or
- a translation start codon in a position that would produce a peptide that did not include the allergen alignment region.

In addition, three RFs produced 8-contiguous amino acid matches to allergens in the COMPARE database. Two of these matches lacked either promoter elements or start codons, as described above. The third, from the translated PMI protein sequence, produced a match to a putative alpha-parvalbumin from frog (DLSDKETT; Hilger et al. 2002). This match is highly unlikely to represent a cross-reactive risk, as demonstrated by numerous previous FSANZ safety assessments[8] and the extensive history of safe use of the PMI protein in crops (Herman et al. 2021).

Given these results, the risk of allergenic proteins with relevance to human safety being produced by novel RFs generated in DP910521 is negligible.

3.4.5.2 Bioinformatic analysis for potential toxicity

The putative peptides encoded by the junction and insert sequences were compared *in silico* to an in-house toxin database (updated in January 2021). This database is a subset of sequences derived from the UniProtKB/Swiss-Prot protein databases, filtered using keywords relating to potential toxicity or adverse health effects. A BLASTP algorithm (v2.11.0+) with a BLOSUM62 scoring matrix and an E-value threshold of 10 was used.

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[6] Evaluation of sequences stop-to-stop codon is a more conservative approach compared to the evaluation of start-to-stop codon sequences.

[7] http://comparedatabase.org/database/

[8] A564, A580, A1001, A1138, A1060, A1202, A1270

No alignments were found between the 925 putative peptides and any known protein toxins. The novel RFs in DP910521 therefore do not present a toxicity concern.

3.5 Conclusion

Corn line DP910521 contains a single copy of the intended DNA insertion, integrated at a specific locus in the corn genome. SbS and sequencing results confirmed that the *pmi*, *mo-pat*, and *cry1B.34* cassettes were inserted with the expected organisation. No backbone sequences from the plasmids used in the transformation are present, including any antibiotic resistance genes.

The inserted DNA is stably inherited and the glufosinate-tolerant phenotype is expressed across several breeding generations of DP910521. Bioinformatics analyses of the new RFs created by the modification did not raise any allergenicity or toxicity concerns.

4 Characterisation and safety assessment of novel substances

In considering the safety of newly expressed substances it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutrient or allergenic effects.

To effectively identify any potential hazards, knowledge of the characteristics, detailed understanding of the biochemical function and phenotypic effects and concentration levels in the edible part of the plant is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

Three novel proteins are expressed in DP910521: the Cry1B.34 insecticidal protein, which provides protection against susceptible lepidopteran pests; the PAT protein, which affords tolerance to the herbicide glufosinate and; the PMI protein, which allows for growth on media containing mannose and acts as a selectable marker during the transformation process.

4.1 Cry1B.34

The crystal (Cry) proteins are a family of pore-forming insecticidal proteins produced by the bacterium *B. thuringiensis.* Upon ingestion of these proteins by the target pest, alkaline conditions and proteases in the insect midgut cause proteolytic cleavage of the protein's protoxin domain and activation of the insecticidal toxin. Binding of the activated Cry protein to receptors on the midgut epithelial cells leads to the formation of ion-conducting pores in the apical membrane of these cells and subsequent cell death (Schnepf et al 1998).

Though Cry proteins have been widely used in GM crops to control insect pests for several decades, the emergence of resistance to these proteins has led to an ongoing need to identify and develop new insecticidal proteins with continuing efficacy against resistant insects. Phylogenetic analyses has established that the diversity of the Cry family of proteins evolved by the independent evolution of three structural domains, and by swapping of domains between toxins (de Maagd et al. 2003). Similarities in the structural domains make it possible to engineer novel chimeric proteins in the laboratory through the exchange of homologous DNA domains between different *cry* genes (Deist et al. 2014).

Cry1B.34 is a chimeric protein encoded by the *cry1B.34* gene. *cry1B.34* is comprised of portions of three *B. thuringiensis* genes: (1) a *cry1B*-class gene; (2) the *cryCa1* gene; and (3) the *cry9Db1* gene (Figure 3). The Cry1B.34 protein is designed to achieve protection against lepidopteran insect species by disrupting the midgut epithelium in these species. The *cry1B.34* gene prepared by the applicant encodes a protein of 1149 amino acids, with an apparent molecular weight of ~129 kDa.

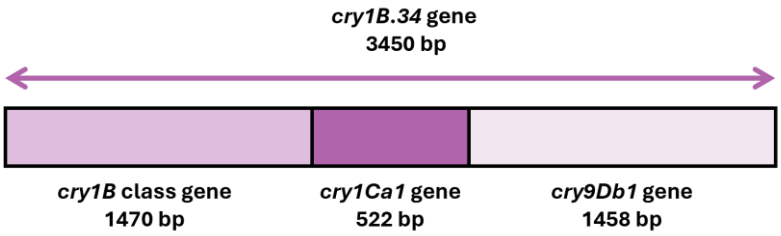

Figure 3. *Representation of the portions of the three B. thuringiensis genes from which the cry1B.34 gene is derived.*

4.1.1 Characterisation of Cry1B.34 expressed in DP910521 and equivalence to a microbially-derived form

Plant-derived Cry1B.34 protein was purified from DP910521 leaf tissue using immunoaffinity chromatography. The purified Cry1B.34 fractions were combined and subsequently concentrated. To obtain sufficient quantities of Cry1B.34 for use in safety studies, Cry1B.34 was also expressed in *Escherichia coli*. The *E. coli*-derived Cry1B.34 was purified by pellet washing and ion exchange chromatography.

The equivalence of the DP910521- and *E.coli*-derived Cry1B.34 proteins must be established before the safety data and conclusions generated using *E. coli*-derived Cry1B.34 can be applied to DP910521-derived Cry1B.34. In order to confirm the identity and equivalence of the DP910521- and *E. coli* derived Cry1B.34, a series of analytical tests were done, the results of which are summarised below.

Molecular weight. Samples of purified DP910521- and *E. coli*-derived Cry1B.34 were subjected to SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. Both proteins migrated with an apparent molecular weight of approximately 129 kDa, as expected.

Immunoreactivity. Western blot analysis with a Cry1B.34-specific antibody showed that the protein being expressed in DP910521 and *E. coli* was indeed Cry1B.34 and that they have equivalent immunoreactivity.

Peptide mapping. DP910521-derived and *E. coli*-derived Cry1B.34 were digested with trypsin or chymotrypsin and analysed by liquid chromatography-mass spectrometry (LC-MS). For DP910521-derived Cry1B.34, the combined tryptic and chymotryptic peptides covered 93.8% of the expected protein sequence (1077 of 1148 amino acids). For *E. coli*-derived Cry1B.34, the peptide coverage was 90.8% of the expected protein sequence (1042 of 1147 amino acids).

N-terminal sequencing. Edman sequencing of the *E.coli*-derived Cry1B.34 identified an N-terminal sequence which matched the first 10 residues of the expected sequence. The N-terminal methionine and adjacent alanine residue were absent from the sequence. For the DP910521-derived Cry1B.34, two N-terminal sequences were examined: one sequence identified by Edman sequencing which matched the first 3 residues of the expected sequence; as well as the N-terminal chymotryptic peptide identified during peptide mapping which matched the first 24 residues of the expected sequence. The N-terminal methionine was absent in both peptides, as expected (Bradshaw et al. 1998; Dummitt et al. 2003), but the adjacent alanine was present.

Glycosylation analysis. SDS-PAGE combined with a colourimetric glycoprotein detection procedure showed that the Cry1B.34 proteins from both DP910521 and *E. coli* were equivalent and that neither is glycosylated. The positive control protein (horseradish peroxidase) showed a band indicative of glycosylation.

Functional activity. The biological activity of *E. coli*-derived Cry1B.34 was evaluated in a 7-day bioassay using *Spodoptera frugiperda* (fall armyworm), a lepidopteran insect which is sensitive to the Cry1B.34 protein. In this assay, *S. frugiperda* larvae were fed a test diet containing 1.563 – 50 ng Cry1B.34 protein/mg diet wet weight or a control diet containing 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer. Mortality and the weight of the surviving organisms were assessed after 7 days.

The results of the bioassay showed that as the concentration of Cry1B.34 in the diet increased from 1.563 to 12.5 ng/mg, mortality increased and the weight of surviving organisms decreased (Table 4). Larvae fed the test diet containing either 25 ng/mg or 50 ng/mg Cry1B.34 showed a mortality of 100%. Larvae fed the control diet showed a mortality of 10%. The results demonstrate that *E. coli*-derived Cry1B.34 is functionally active against the target insect *S. fruigiperda*.

Table 4: Bioactivity of microbially-derived Cry1B.34 in a diet fed to insect larvae

Treatment description	Cry1B.34 concentration (ng/mg diet)	Number of observations	Total number of surviving organisms	Mortality (%)	Weight of surviving organisms (mg)	
					Mean ± standard deviation	Range
Buffer control diet	0	20	18	10	36.6 ± 7.5	22.5 – 49.0
Test diet	1.563	20	17	15	0.6 ± 0.4	0.2 – 1.7
	3.125	20	15	25	0.3 ± 0.3	0.1 – 1.3
	6.25	20	10	50	0.1 ± 0.1	0 – 0.2
	12.5	20	3	85	0.1 ± 0.1	0.1 – 0.2
	25	19	0	100	-	-
	50	19	0	100	-	-

The results outlined in this section demonstrated that *E.coli*-derived Cry1B.34 is structurally and biochemically equivalent to DP910521-derived Cry1B.34. The biological activity of *E. coli*-derived Cry1B.34 was demonstrated in an insect bioassay and based on the structural and biochemical equivalence to DP910521-derived Cry1B.34, the two proteins are expected to be functionally equivalent. It can be concluded that *E. coli*-derived Cry1B.34 is a suitable surrogate for use in the safety studies described in Section 4.1.2.

4.1.2 Safety of the introduced Cry1B.34

Data were provided to assess the potential allergenicity and toxicity of Cry1B.34.

Bioinformatic analyses of Cry1B.34

In silico analyses were performed to compare the Cry1B.34 amino acid sequence to known allergenic proteins in the COMPARE database (January 2021). The analyses were performed as described in section 3.4.5.1, except the E-value threshold for the FASTA search was set to 0.0001. The search did not identify any known allergens with homology to Cry1B.34. No alignments met or exceeded the threshold of ≥ 35% over 80 amino acids and no contiguous eight amino acid peptide matches were shared between the Cry1B.34 sequence and proteins in the allergen database.

To assess the similarity of Cry1B.34 to known toxins, the applicant provided the results of *in silico* analyses comparing the Cry1B.34 amino acid sequence to proteins identified as “toxins” in the same in-house database described in section 3.4.5.2 (January 2021). A BLASTP algorithm was used with a BLOSUM62 scoring matrix, the low complexity filtering

was turned off and the E-value threshold set to 0.0001. The search did not identify any known toxins with homology to Cry1B.34.

Susceptibility of Cry1B.34 to digestion with pepsin and pancreatin

E. coli-derived Cry1B.34 (test substance) was incubated at 37°C in a simulated gastric fluid (SGF) system containing pepsin (10 units enzyme/µg protein) at an acidic pH of ~1.2. Incubation of the test substance was for 0, 0.5, 1, 2, 5, 10, 20, and 60 min. Bovine serum albumin (BSA; positive control) and β-lactoglobulin (negative control) were incubated in SGF for 0, 1, and 60 min. In addition, controls containing either SGF without protein, Cry1B.34 in buffer, or Cry1B.34 in a gastric control solution that did not contain pepsin were each incubated for 60 min. The extent of digestion was visualised using GelCode Blue Stain Reagent on an SDS-PAGE gel, followed by Western blotting.

The results from the pepsin digestion showed that by 0.5 min, there was no intact Cry1B.34 remaining in the reaction mix. A ~20 kDa band was evident in the reaction mixture for the first 2 min of digestion, but was fully digested within 5 min. Some lower molecular weight bands were also detectable by SDS-PAGE (but not by Western blot) throughout the 60 min time course. The BSA control was digested within 1 min in SGF and the β-lactoglobulin control remained intact in the reaction mix after 60 min. Cry1B.34 remained intact after 60 min in buffer and in the gastric solution without pepsin. These results indicate that Cry1B.34 is rapidly digested by pepsin.

E. coli-derived Cry1B.34 protein was also incubated with pancreatin[9] (20 µg enzyme/µg protein) at 37°C in a simulated intestinal fluid (SIF) system at a neutral pH of ~7.5. Incubation of Cry1B.34 was for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 min. BSA (positive control) and β-lactoglobulin (negative control) were incubated in SIF for 0, 1, and 60 min. In addition, controls containing either SIF without protein, Cry1B.34 in buffer, or Cry1B.34 in a gastric control solution that did not contain pancreatin were each incubated for 60 min. The extent of digestion was visualised using GelCode Blue Stain Reagent on an SDS-PAGE gel, followed by Western blotting.

The results from the pancreatin digestion showed that intact Cry1B.34 was digested within 0.5 min, as judged by SDS-PAGE, and within 1 min as judged by Western blot. Smaller fragments <75 kDa remained in the reaction mix throughout the 60 min incubation period. The BSA control remained undigested after 60 min in SIF and the β-lactoglobulin control was digested within 60 min in SIF. Cry1B.34 remained intact after 60 min in buffer and in the intestinal solution without pancreatin. These results indicated that full length Cry1B.34 is rapidly digested by pancreatin, though some smaller fragments remain resistant to digestion.

Cry1B.34 was also subjected to a sequential digestion with pepsin (SGF, as above) followed by pancreatin (SIF, as above). Cry1B.34 was first incubated with SGF for 10 min, then with SIF over a 0-60 min time course. The low molecular weight fragments (~2-5 kDa) that were evident after 10 minutes of SGF digestion were rapidly digested within 0.5 min of sequential SIF digestion. Taken together, these results indicate that Cry1B.34 would be fully degraded by gastric and intestinal enzymes in the human digestive system.

Bioactivity of Cry1B.34 after exposure to heat

The thermal stability of Cry1B.34 was evaluated by assessing the functional activity of the heat-treated Cry1B.34 protein in a 7-day insect bioassay. *E.coli*-derived Cry1B.34 was incubated for 30-35 minutes at 25°C, 50°C, 75°C, or 95°C before incorporation into an artificial diet for *S. frugiperda* larvae (see section 4.1.1). Control diets contained either buffer

[9] Pancreatin is a mixture of proteolytic enzymes

(negative control), or unheated Cry1B.34 (positive control). The test diets and the unheated control diet contained a target concentration of 25 ng Cry1B.34 protein per mg diet wet weight. Each diet was provided orally to 20 individual *S. frugiperda* larvae for a total of 7 days, with refeeding occurring on day 4. Mortality and the weight of surviving larvae were assessed after day 7. Enzyme-linked immunosorbent assay (ELISA) analysis confirmed the dose and homogeneity of the Cry1B.34 protein during the assay.

Table 5: Bioactivity of heat-treated Cry1B.34 in a diet fed to insect larvae

Treatment description	Incubation condition	Number of observations	Total number of surviving organisms	Mortality (%)	p-value[a]	Weight of surviving organisms (mg)	
						Mean ± standard deviation	Range
Buffer control diet	-	20	20	0	-	36.7 ± 13.9	2.3 – 50.1
Unheated control diet	-	20	0	100	-	-	-
Test diet	25°C	20	0	100	1.0000	-	-
	50°C	20	1	95	0.5000	0.100[b]	-
	75°C	20	20	0	<0.0001	13.8 ± 7.88	0.2 – 34.7
	95°C	20	20	0	<0.0001	41.1 ± 8.55	24.0 – 59.4

a. The p-value is derived from a Fisher's exact test comparing the test diets to the unheated control diet. A p-value of <0.05 indicates a statistically significant difference. b. The reported mean is the weight of the single surviving larva.

The results demonstrated that when heated to temperatures of 75-95°C for ~30 minutes, the the bioactivity of Cry1B.34 was effectively abolished, with 0% mortality observed for the larvae fed diets containing Cry1B.34 heat-treated at either 75°C or 95°C (Table 5). Fisher's exact test was used to determine whether the mortality rate of *S. frugiperda* that had been fed diets containing heated Cry1B.34 was smaller than that of those fed the unheated control diet. For Cry1B.34 heated to 75°C or 95°C, the decrease in activity against *S. frugiperda* larvae was statistically significant (p-value <0.05) compared to the unheated control. Cry1B.34 heat-treated at either 25°C or 50°C did not have a statistically significant decrease in activity compared to the unheated control (Table 5). These data indicate that Cry1B.34 is heat labile at temperatures ≥ 75°C.

14-day acute oral toxicity study

Although the bioinformatic analyses, digestibility tests and heat susceptibility tests did not raise any safety concerns, a 14-day acute oral toxicity study in mice using *E.coli*-derived Cry1B.34 was submitted by the applicant as additional supporting information.

The potential acute toxicity of Cry1B.34 protein (82% purity) was assessed in mice following a single oral exposure. Cry1B.34 protein was administered orally via gavage at doses of 0 or 5000 mg/kg body weight to mice (6/sex/group). The vehicle/negative control was water and another equivalent group of mice received BSA protein (5000 mg/kg body weight), as a comparative control. The dose volume was 20 mL/kg body weight for all formulations.

The vehicle control, BSA and Cry1B.34 protein formulations were administered on day 1 in three split doses, separated by 4 hours. The mice were fasted prior to and throughout dosing. Mice were housed individually (males) or in pairs (females) under standard laboratory conditions. A 2-week observation period followed dosing. Mice were observed twice daily for animal health and daily for clinical condition. Additional clinical observations were made on day 1 (prior to administration of each formulation dose; and 30 minutes and 1-2 hours after each dose administration). Body weights were recorded on days 1, 2, 3, 5, 8 and 15. All animals were euthanised on day 15 and subject to a gross pathological examination of an extensive range of organs and tissues. The gastrointestinal tracts were

prepared for histopathological examination but were later discarded as no microscopic evaluations were required.

All animals survived to study termination. There were no treatment related effects on body weight, clinical or gross observations. It was concluded that the oral LD_{50} of Cry1B.34 protein was greater than 5000 mg/kg body weight.

4.1.3 Conclusion

A range of characterisation studies were performed on plant-derived Cry1B.34 confirming its identity, structure and biochemistry as well as equivalence of the corresponding protein derived from a bacterial expression system. The microbially-derived Cry1B.34 was also shown to be functional. Bioinformatic analyses showed Cry1B.34 did not share any meaningful homology with any known allergens or toxins. Cry1B.34 was heat labile at ≥ 75°C and susceptible to digestion by gastrointestinal enzymes. Additionally, an acute oral toxicity study in mice did not result in any treatment-related adverse effects. Taken together this indicates that the Cry1B.34 protein is unlikely to be toxic or allergenic to humans.

4.2 PAT

The *mo-pat* gene in DP910521 encodes the protein phosphinothricin N-acetyltransferase (PAT), which enzymatically inhibits phosphinothricin (PPT) (Strauch et al. 1988; Wohlleben et al. 1988). PPT is the active constituent of glufosinate ammonium herbicides and acts by irreversibly inhibiting the endogenous plant enzyme glutamine synthetase. This enzyme is involved in amino acid biosynthesis in plant cells and its inhibition causes accumulation of ammonia, leading to plant death. In glufosinate-tolerant GM plants, the introduced PAT enzyme chemically inactivates PPT by acetylation of the free ammonia group to produce N-acetyl glufosinate, allowing plants to continue amino acid biosynthesis in the presence of the herbicide (Hérouet et al. 2005).

The PAT protein has a long history of safe use in agriculture, and is present in a number of crops currently in commercial use (CERA-ILSI 2016). There have been no credible reports of adverse effects on human health since it was introduced into food.

The *mo-pat* gene in DP910521 has been codon optimised for expression in corn. The deduced amino acid sequence from translation of the *mo-pat* gene is identical to that produced from the *pat* gene in the source organism *S. viridochromogenes*. Both genes encode a 183 amino acid protein with a calculated molecular weight of ~21 kDa, and also share 85% amino acid identity with PAT protein encoded by the *bar* gene from *S. hygroscopicus*. The two PAT proteins are regarded as equivalent (OECD 1999): both exhibit a high degree of enzyme specificity, recognising and detoxifying only PPT (Wehrmann et al. 1996).

4.2.1 Safety of the introduced PAT

The PAT protein (expressed from either the *pat* or *bar* genes) has been considered in 34 previous FSANZ safety assessments, including 15 in corn. These assessments, together with the published literature, have established the safety of PAT and confirm that it does not raise toxicity or food allergenicity concerns in humans (Hérouet et al. 2005; Delaney et al. 2008; Hammond et al. 2011; CERA-ILSI 2016).

In previous FSANZ assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans. Since the sequence of the protein expressed in DP910521 is identical to the previous PAT sequences assessed by FSANZ, no further safety evaluation is required other than the examination of updated

bioinformatics searches.

Updated bioinformatic studies (January 2022) for PAT that looked for amino acid sequence similarity to known protein allergens and toxins were provided by the applicant with their submission for application A1270 (FSANZ, 2023). The results do not alter conclusions reached in previous assessments.

4.2.2 Conclusion

The data provided by the applicant confirms the PAT expressed in DP910521 is identical to previously assessed PAT proteins. Updated bioinformatic analyses confirm that PAT has no similarity with known allergens or toxins that is of significance or concern.

4.3 PMI

The *pmi* gene in DP910521 encodes the enzyme phosphomannose isomerase (PMI), which catalyses the interconversion of mannose 6-phosphate and fructose 6-phosphate. Expression of PMI allows plant cells to use mannose as a source of carbon, which assists with the identification of transformed cells (Negrotto et al. 2000).

The *pmi* gene encodes a 391 amino acid protein with a calculated molecular weight of ~43 kDa.

4.3.1 Safety of the introduced PMI

The PMI protein has been previously assessed and approved by FSANZ in 7 corn lines and one rice line[10].These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans. Since the PMI protein expressed in DP910521 is identical in sequence to the PMI protein expressed in previously assessed corn and rice lines, no further safety evaluation is required other than the examination of updated bioinformatics searches.

Updated bioinformatic studies (January 2022) for PMI that looked for amino acid sequence similarity to known protein allergens and toxins were provided by the applicant with their submission for application A1270 (FSANZ, year). The results do not alter conclusions reached in previous assessments.

4.3.2 Conclusion

The data presented by the applicant confirms the PMI expressed in DP910521 is identical to previously assessed PMI proteins. Updated bioinformatic analyses confirm that PMI has no similarity with known allergens or toxins that is of significance or concern.

4.4 Protein expression levels in DP910521

For analysis of the expression levels of the Cry1B.34, PAT and PMI proteins in DP910521, tissues were collected from six field-trial sites in representative corn-producing regions of the United States and Canada during the 2020 growing season[11]. Tissues were collected at varying stages of growth (see Figure 4 for a summary of corn growth stages). Tissues were lyophilised, homogenised (except pollen samples) and stored frozen until analysis.

[10] Corn lines 5307 (Application A1060; FSANZ 2012), MIR162 (Application A1001; FSANZ 2008a), 3272 (Application A580; FSANZ 2008b), MIR604 (Application A564; FSANZ 2006), DP23211 (A1202; FSANZ 2020), DP51291 (Application A1270; FSANZ 2023), and DP915635 (Application A1272; FSANZ 2023b), and rice line GR2E (Application A1138; FSANZ 2017)

[11] Field sites for testing protein expression levels were in the following United States and Canadian states – Iowa, Illinois, Nebraska, Pennsylvania, Texas, and Ontario.

Cry1B.34, PAT and PMI were extracted from tissues using standard methods and their expression levels were quantified in each tissue using a quantitative ELISA. For each tissue analysed, four samples were processed from each of the six field-trial sites. Samples from both glufosinate-treated and non-treated DP910521 were collected.

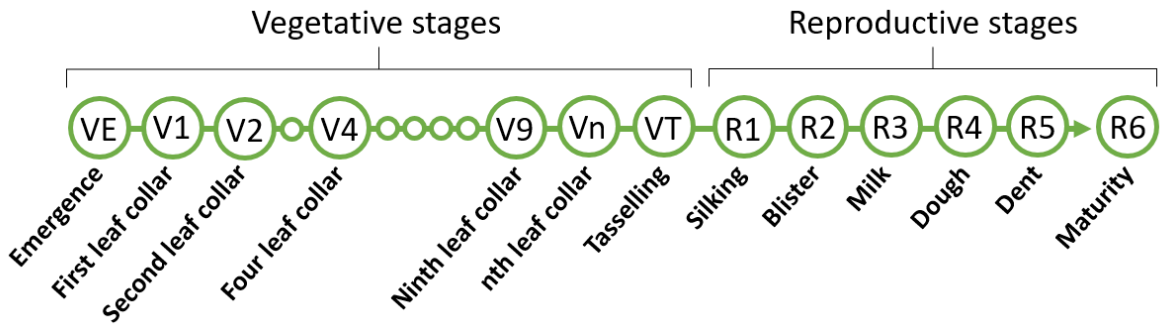

Figure 4. *Stages of corn growth. Grain is harvested at maturity (R6).*

Results from the ELISA showed that for Cry1B.34, the highest expression levels in herbicide-treated DP910521 were in leaf, which is the target tissue for lepidopteran insect consumption (Figure 5a). Cry1B.34 was expressed at a very low level in grain and pollen (6.1 ng/mg and 0.2 ng/mg, respectively).

PAT was expressed at a relatively uniform level throughout the herbicide-treated DP910521, except in grain, where its expression was low (11 ng/mg; Figure 5a). PMI had the lowest expression levels in all tissues, and was expressed at particularly low levels in root and grain (5.3 ng/mg; Figure 5a).

Similar levels of all proteins were detected in DP910521 not treated with glufosinate (Figure 5b). For the full set of expression data, including standard errors and ranges, refer to the Application dossier[12] (pages 127 – 128).

4.5 Herbicide metabolites

FSANZ has assessed the herbicide metabolites for glufosinate in GM crops in multiple previous applications. These previous assessments indicate the spraying of DP910521 with glufosinate ammonium would result in the same metabolites that are produced in non-GM corn sprayed with the same herbicide. As no new glufosinate metabolites would be generated in corn event DP910521, further assessment is not required.

[12] https://www.foodstandards.gov.au/food-standards-code/applications/a1281-food-derived-herbicide-tolerant-and-insect-protected-corn

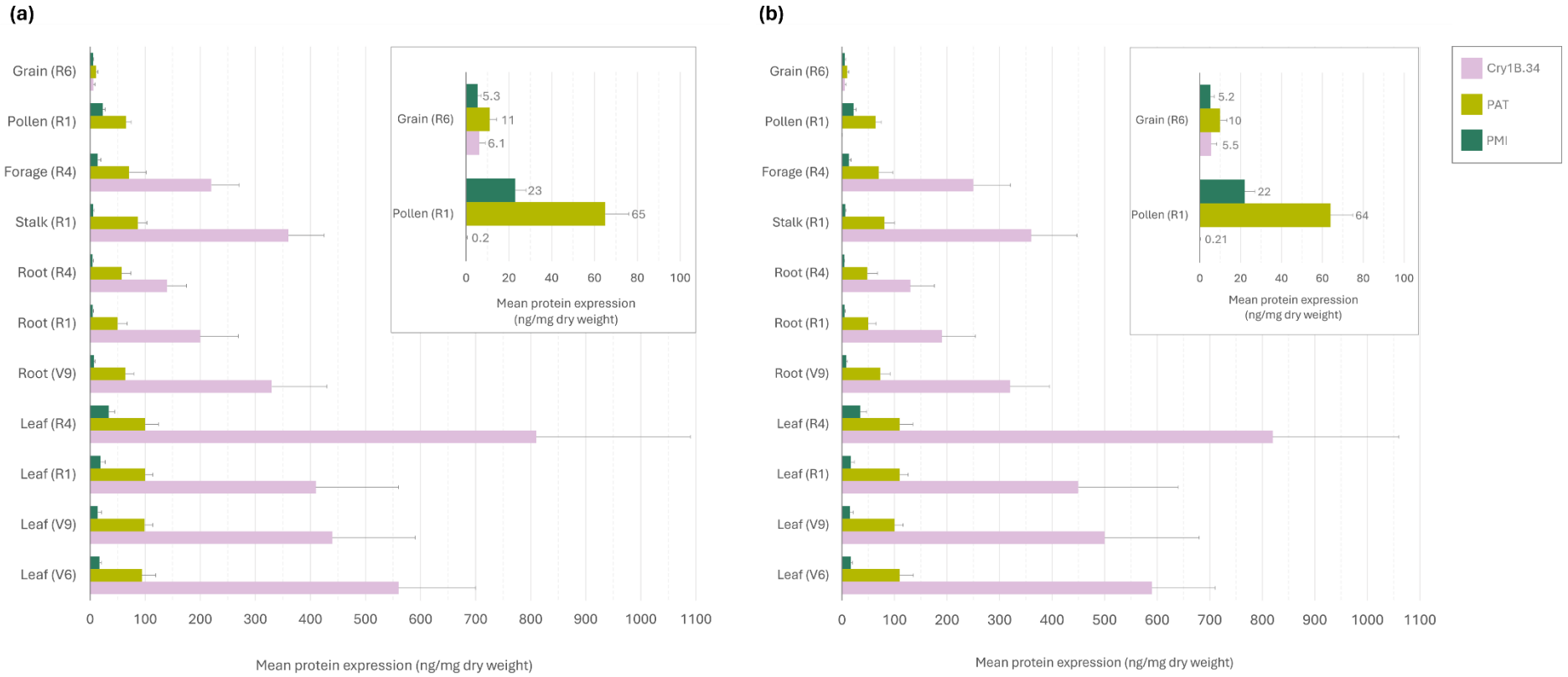

Figure 5: *Mean expression levels (ng/mg dry weight) of the Cry1B.34, PAT, and PMI proteins in* **(a)** *glufosinate-treated and* **(b)** *untreated DP910521 tissues. Insets in both panels show a more detailed view of the expression levels in grain and pollen. Error bars represent standard deviation.*

5 Compositional analysis

The main purpose of compositional analyses is to determine if, as a result of the genetic modification, any unexpected change has occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analyses can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The classic approach to the compositional analyses of GM food is a targeted one. Rather than analysing every possible constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors such as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health.

5.1 Key components

The key components to be analysed for the comparison of GM and conventional corn are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Maize (OECD 2002), and include: proximates (protein, fat, fibre, ash, carbohydrates), amino acids, fatty acids, minerals, vitamins and the anti-nutrients phytic acid, raffinose, furfural and the phenolic acids ferulic acid and *p*-coumaric acid.

5.2 Study design

DP910521 corn, a non-GM control corn of similar genetic background, and a total of 18 non-GM commercial reference corn lines[13] were grown and harvested from eight field trial sites in the United States and Canada during the 2020 growing season[14]. The sites were representative of corn growing regions suitable for commercial production.

The field sites were established in a randomised complete block design with four replicates per site. Each block contained DP910521 corn, non-GM control corn, and four reference corn lines selected from the 18 non-GM reference lines. Plants were grown under agronomic field conditions typical for each growing region. A herbicide treatment of glufosinate was applied to DP910521.

At maturity (R6 growth stage), grain was harvested from all plots, with reference and control grain collected prior to glufosinate-treated DP910521 samples to minimise the potential for contamination. Following harvest, samples were chilled before being transferred to a freezer (< -10°C) and shipped frozen to an analytical laboratory with full identity labelling. Compositional analyses were performed based on internationally recognised procedures including official methods specified by the Association of Official Analytical Chemists (AOAC), the Analytical Oil Chemists' Society (AOCS), methods specified by the manufacturer of the equipment used for analysis, or other published scientific methods.

[13] The 18 reference corn lines: P0506, XL5513, P0574, PB5646, P0760, G07F23, 207-27, BK5883, P0843, BKXL-5858, 209-50, P0928, P0993, 6046, P1093, DKC60-84, G10T63, BK6076

[14] The location of the eight field trial sites: one site in each of Iowa, Nebraska, Pennsylvania, Texas, Wisconsin, and Ontario; two sites in Illinois.

A total of 70 analytes in grain were assessed (see Figure 6 for a complete list, not including moisture). For 4 of these analytes (listed in grey in Figure 6) all samples of both DP910521 and the non-GM control gave results below the assay lower limit of quantification (LLOQ) and were therefore not analysed statistically.

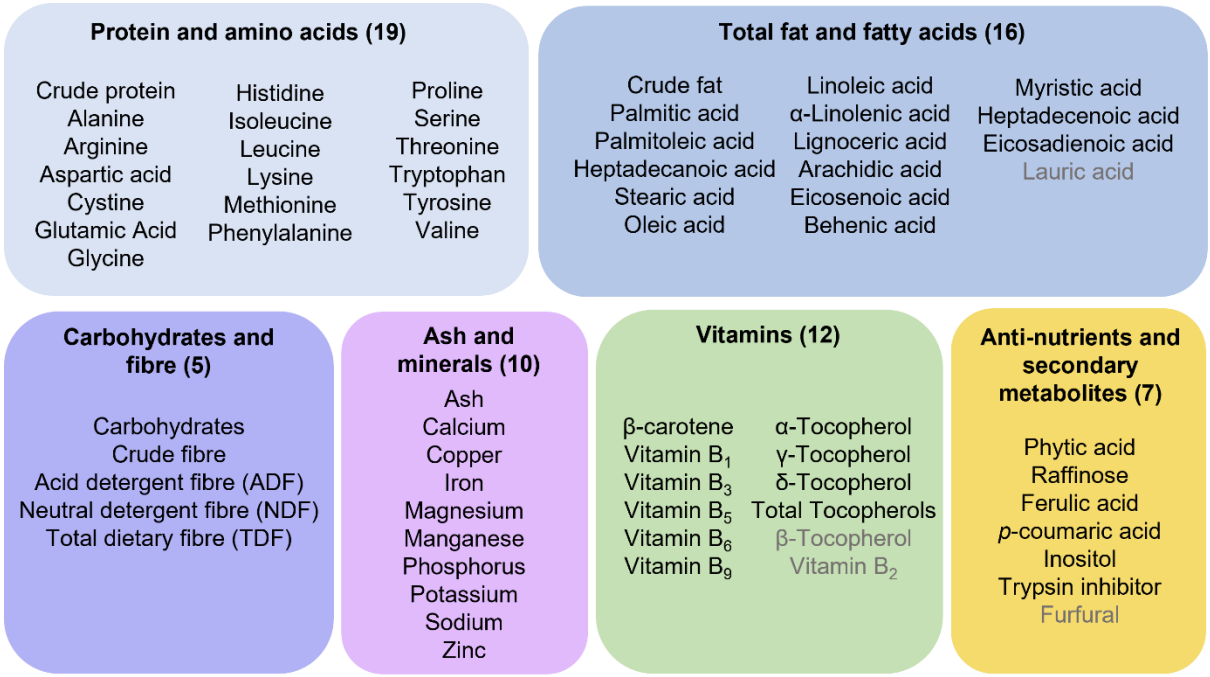

Figure 6. *Analytes measured in DP910521 grain samples. Analytes listed in grey text had all samples below the LLOQ and were excluded from statistical analysis. The analytes listed in black text, as well as moisture, were analysed fully.*

For the remaining 66 analytes, 'descriptive statistics' (mean, range and 95% confidence interval) were generated. For 62 of these analytes, where both DP910521 and the non-GM control corn had <50% of samples below the LLOQ, a linear mixed model analysis of variance was applied for combined data and locations, covering the eight replicated field trial sites. The mixed model analysis was also applied to the data from each site separately. For the remaining 4 analytes (myristic acid, heptadecenoic acid, eicosadienoic acid, and δ-tocopherol), >50% of either DP910521 or the control corn samples were below the LLOQ. For these analytes, Fisher's exact test was used to assess whether there was a significant difference in the proportion of samples below the LLOQ between the two corn lines across sites. Individual site analyses were not performed for these analytes.

In assessing the statistical significance of any difference between DP910521 and the conventional control, a p-value of 0.05 was used. A further adjusted p-value was determined using the false discovery rate (FDR) method, as a consideration of the chance of false positives being observed with the testing due to the multiple analytes being analysed (Benjamini and Hochberg 1995). In cases where the raw p-value was <0.05 but the FDR-adjusted p-value was >0.05, the difference was considered likely to be a false positive.

Any statistically significant differences between DP910521 and the control were compared to tolerance intervals derived from an in-house database containing compositional analyses from 184 non-GM commercial corn lines cultivated across 185 unique environments in North and South America from 2003-2019. Tolerance intervals are expected (with 95% confidence) to contain at least 99% of the values for corresponding analytes of the conventional corn population (Hong et al. 2014). In addition, compositional data from the non-GM reference varieties grown concurrently in the same trial as DP910521 and the control were combined

across all sites and used to calculate an in-study reference range for each analyte. This reference range is useful to define the variability in corn varieties grown under the same agronomical conditions. Finally, the natural variation of analytes from publicly available data was also considered (Watson 1982; OECD 2002; Codex 2019; Lundry et al. 2013; Cong et al. 2015; AFSI 2021). These data ranges assist with determining whether any statistically significant differences are likely to be biologically meaningful.

5.3 Analyses of key components in grain

Of the 66 analytes for which mean values were provided (Figure 6), there were 4 for which there was a statistically significant difference ($p < 0.05$) between herbicide-treated corn line DP910521 and the non-GM control: ash, zinc, vitamin B_1, and *p*-coumaric acid. A summary of these 4 analytes is provided in Figure 7. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the Application dossier[15] (pages 130-149).

For the 4 analytes for which a statistically significant difference was found, all had FDR-adjusted p-values of >0.05 (Figure 7), suggesting that the differences in these analytes were likely to be false positives. In addition, for each of these 4 analytes, the deviation of the DP910521 mean from the control mean was less than 7% (Figure 7a). As can be observed in Figure 7 (panels b-e), the DP910521 mean for each of these 4 analytes was within the range of values observed for the control, indicating that DP910521 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. For all 4 analytes, the observed DP910521 means fall within the natural variability represented by the tolerance interval, in-study reference range and publicly available range (purple shaded area, dark grey and light grey bars, respectively, in Figure 7, b-e). The differences reported here are therefore consistent with the normal biological variability that exists in corn.

Overall, the compositional data support the conclusion that no biologically significant differences exist in the levels of key constituents in DP910521 when compared with conventional non-GM corn cultivars already available in agricultural markets. Grain from DP910521 can therefore be regarded as equivalent in composition to grain from conventional non-GM corn.

[15] https://www.foodstandards.gov.au/food-standards-code/applications/a1281-food-derived-herbicide-tolerant-and-insect-protected-corn

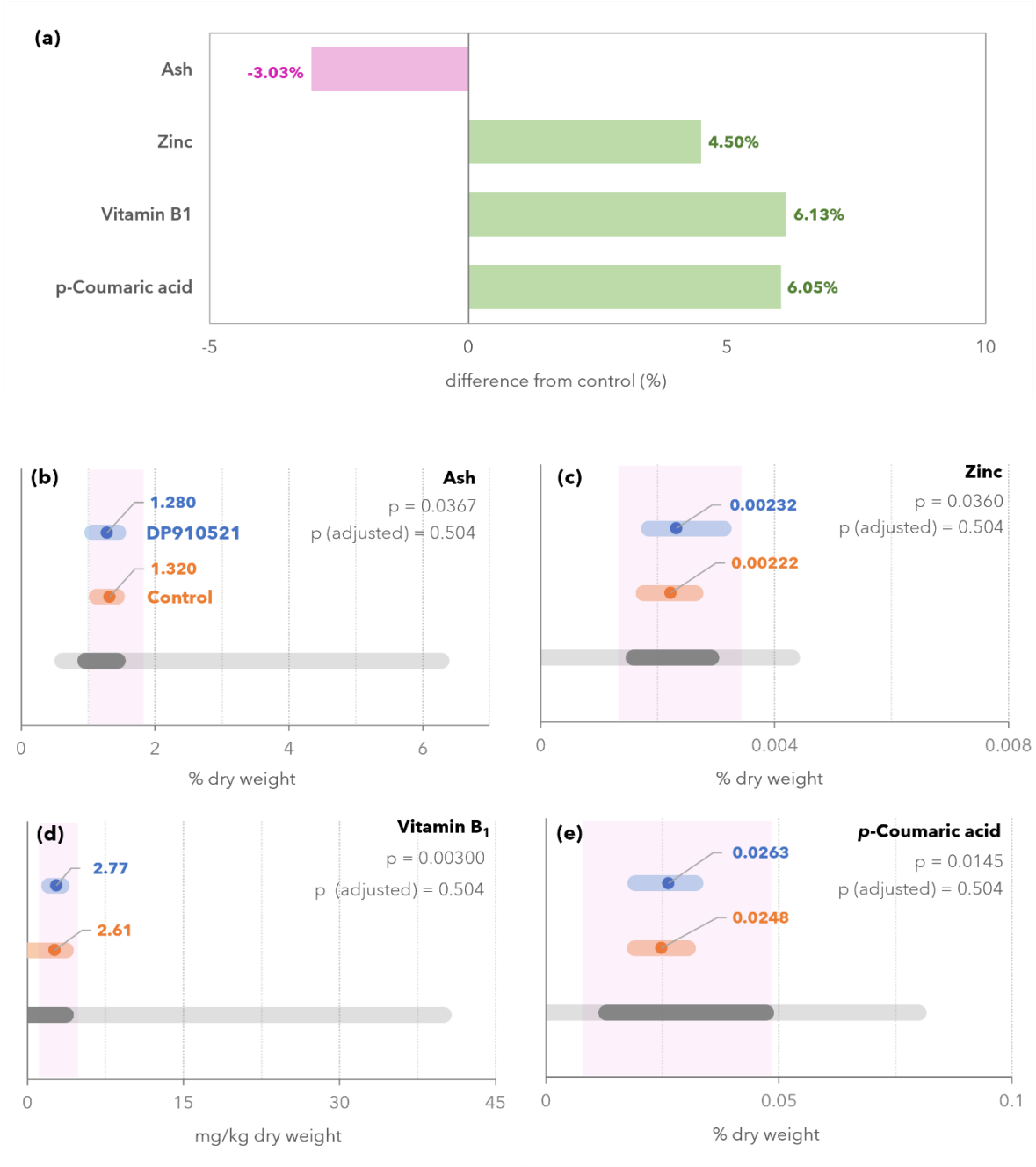

Figure 7. *Visual summary of statistically significant compositional differences between DP910521 and the conventional control corn.* **(a)** *Percentage deviation of the mean DP910521 value from the mean control value for each of the 4 analytes for which a statistically significant difference was found.* **(b) – (e)** *Measured means (dots) and ranges (coloured bars) for DP910521 (blue) and the conventional control (orange) for the 4 analytes as labelled. The light and dark grey bars represent the in-study reference range and publicly-available range of values, respectively, for each analyte. The purple shaded range represents the tolerance interval for each analyte. Note that the x-axes vary in scale and unit for each component.*

6 Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food, such as that presented in Section 5.

Where a GM food has been shown to be compositionally equivalent to conventional cultivars, the evidence to date indicates that feeding studies using target livestock or other animal species will add little to the safety assessment (OECD 2003; Bartholomaeus et al. 2013). If the compositional analysis indicates biologically significant changes, either intended or unintended, to the levels of certain nutrients in the GM food, additional nutritional studies should be undertaken to assess the potential impact of the changes on the whole diet.

DP910521 is the result a genetic modification to confer tolerance to the herbicide glufosinate and protection against lepidopteran insect pests, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modification has not altered the nutrient composition DP910521 compared with conventional non-GM corn cultivars. The introduction of food derived from DP910521 into the food supply is therefore expected to have negligible nutritional impact.

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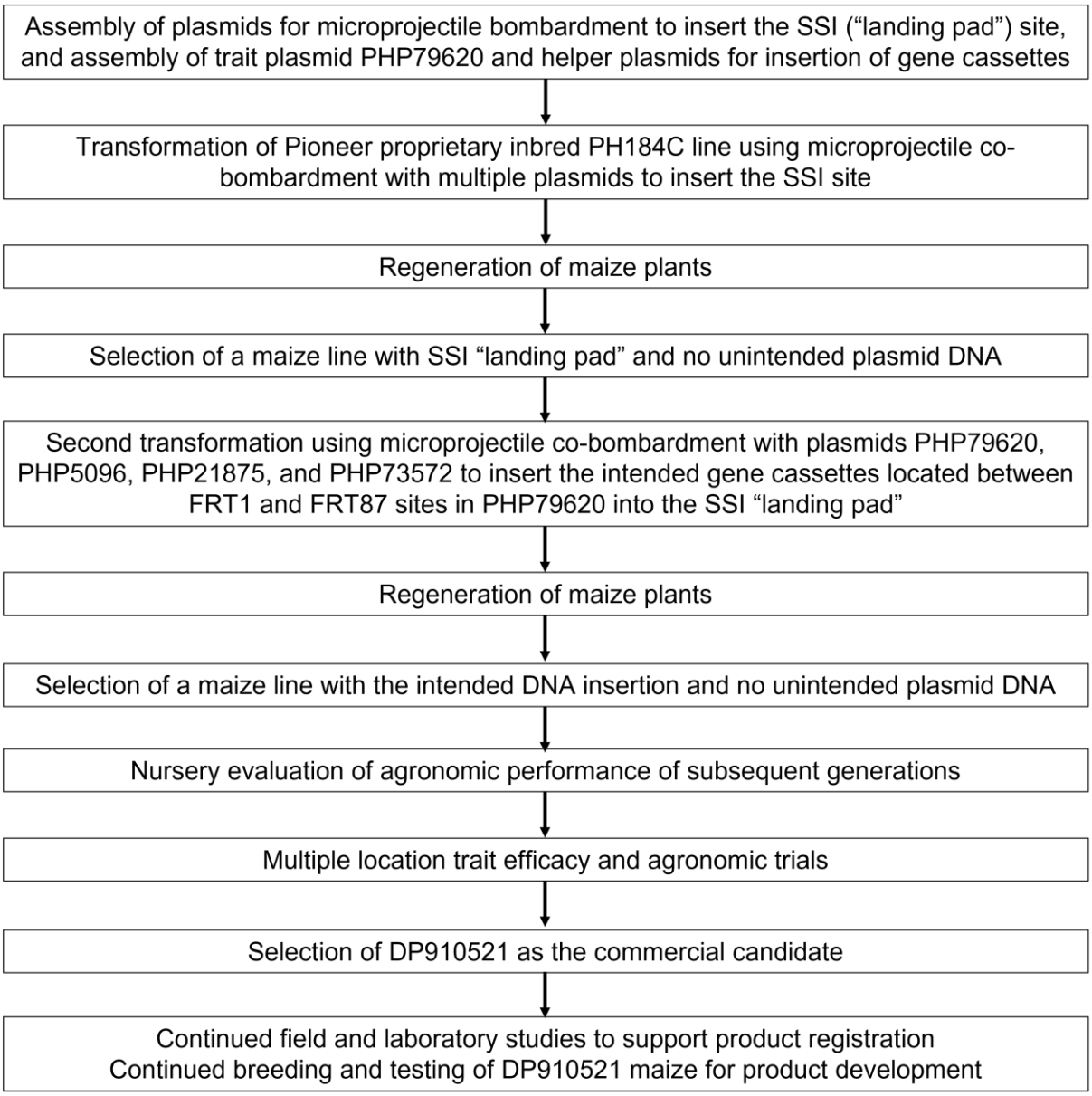
Appendix 1

Development of DP910251



Appendix 2

PHP79620-derived genetic elements in DP910521

Genetic Element	Relative position	Size (bp)	Description, Source and Reference
FRT1	1-48	48	Flippase recombination target site from *Saccharomyces cerevisiae* (Proteau et al. 1986)
Intervening Sequence	49-66	18	DNA sequence used for cloning
***pmi* gene cassette**			
pmi	67 -1,282	1,216	Phosphomannose isomerase gene from *Escherichia coli* including 5' and 3' untranslated regions (UTR) (Negrotto et al. 2000) as described below: • 5' UTR at bp 67-70 (4 bp long) • Coding sequence at bp 71-1,246 (1,176 bp long) • 3' UTR at bp 1,247-1,282 (36 bp long)
Intervening Sequence	1,283 - 1,292	10	DNA sequence used for cloning
pinII Terminator	1,293 - 1,603	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al. 1989; Keil et al. 1986)
Intervening Sequence	1,604- 1,613	10	DNA sequence used for cloning
Z19 Terminator	1,614- 2,355	742	Terminator region from the *Zea mays* 19-kDa zein gene (GenBank accession KX247647; Dong et al. 2016)
Intervening Sequence	2,356 - 2,558	203	DNA sequence used for cloning
***mo-pat* gene cassette**			
os-actin Promoter	2,559 - 4,240	1,682	Promoter region from the O*ryza sativa* (rice) actin gene (GenBank accession CP018159; GenBank accession EU155408.1)
os-actin Intron	4,241- 4,709	469	Intron region from the *O. sativa* actin gene (GenBank accession CP018159; GenBank accession EUl55408.1)
Intervening Sequence	4,710- 4,724	15	DNA sequence used for cloning
mo-pat	4,725 - 5,276	552	Maize-optimized phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes* (Wohlleben et al. 1988)
Intervening Sequence	5,277 - 5,294	18	DNA sequence used for cloning
CaMV35S Terminator	5,295 - 5,488	194	35S terminator region from the cauliflower mosaic virus genome (Franck et al. 1980; Guilley et al. 1982)
Intervening Sequence	5,489 - 5,509	21	DNA sequence used for cloning
*lox*P	5,510- 5,543	34	Bacteriophage Pl recombination site recognized by Cre recombinase (Dale and Ow 1990)
Intervening Sequence	5,544 - 5,639	96	DNA sequence used for cloning

sb-ubi Terminator	5,640 - 6,223	584	Terminator region from the *Sorghum bicolor* (sorghum) ubiquitin gene (Phytozome gene ID Sobic.004G049900.l; US Patent 9725731 Abbitt 2017)
Intervening Sequence	6,224 - 6,264	41	DNA sequence used for cloning
sb-gkaf Terminator	6,265 - 6,728	464	Terminator region from the *S. bicolor* γ-kafirin gene (de Freitas et al. 1994)
Intervening Sequence	6,729- 6,761	33	DNA sequence used for cloning
*att*Bl	6,762 - 6,785	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway® cloning system (Hartley et al. 2000; Katzen 2007)
Intervening Sequence	6,786 - 6,823	38	DNA sequence used for cloning
cry1B.34 gene cassette			
MMV Enhancer	6,824-7,010	187	Enhancer region from the mirabilis mosaic virus genome (Dey and Maiti, 1999)
Intervening Sequence	7,011- 7,020	10	DNA sequence used for cloning
MMV Enhancer	7,021- 7,207	187	Enhancer region from the mirabilis mosaic virus genome (Dey and Maiti 1999)
Intervening Sequence	7,208 - 7,230	23	DNA sequence used for cloning
LLDAV Promoter	7,231- 8,456	1,226	Promoter region from the lamium leaf distortion- associated virus genome (Zhang et al. 2008)
Intervening Sequence	8,457 - 8,474	18	DNA sequence used for cloning
zm-i6 Intron	8,475 - 9,078	604	Intron region from the *Z. mays* translation initiation factor 6 gene (Phytozome gene ID GRMZM2G318475; US Patent 10344290 Diehen et al. 2019)
Intervening Sequence	9,079 - 9,084	6	DNA sequence used for cloning
zm-extensin 5'UTR	9,085 - 9,151	67	5' untranslated region from a *Z. mays* extensin gene (GenBank accession NM001111947.2; UniProt accession P14918)
Intervening Sequence	9,152 - 9,163	12	DNA sequence used for cloning
*cry*1B.34	9,164- 12,613	3,450	Chimeric gene comprised of sequences from a *cry*1B-class gene, the *cry*1Ca1 gene, and the *cry*9Db1 gene, all derived from *Bacillus thuringiensis* (WO Patent 2016061197 Izumi Wilcoxon and Yamamoto 2016); (GenBank accession CAA30396.1; US Patent 7541517 Flannagan and Abad 2009), respectively) as described below: • *cry*1B-class at bp 9,164 - 10,633 (1,470 bp long) • *cry*1Ca1 at bp 10,634 - 11,155 (522 bp long) • *cry*9Db1 at bp 11,156- 12,613 (1,458 bp long)
Intervening Sequence	12,614- 12,619	6	DNA sequence used for cloning

os-ubi Terminator	12,620 - 13,569	950	Terminator region from the *O. sativa* ubiquitin gene (Phytozome gene ID LOC Os06g46770.1; Wang et al. 2000)
Intervening Sequence	13,570 - 13,613	44	DNA sequence used for cloning
*att*B3	13,614 - 13,634 (complementary)	21	Bacteriophage lambda integrase recombination site (Cheo et al. 2004)
Intervening Sequence	13,635 - 13,869	235	DNA sequence used for cloning
FRT87	13,870 - 13,917	48	Modified flippase recombination target site derived from *S. cerevisiae* (Tao et al. 2007)