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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Safety assessment – Application A1272

Food derived from herbicide-tolerant and insect-protected corn line DP915635

Executive summary

Background

Application A1272 seeks approval for the sale and use of food derived from corn line DP915635 that has been genetically modified (GM) for tolerance to the herbicide glufosinate and protection from coleopteran insect pests, primarily western corn rootworm (WCR).

Protection against corn rootworm is conferred by the expression of the *ipd079Ea* gene, from the fern *Ophioglossum pendulum*, encoding the IPD079Ea protein. This protein causes damage to the midgut epithelium of corn rootworm larvae, resulting in insect death. Tolerance to the herbicide glufosinate is achieved by the expression of the maize-optimised *mo-pat* gene, derived from the bacterium *Streptomyces viridochromogenes*, encoding the enzyme phosphinothricin acetyltransferase (PAT). DP915635 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 IPD079Ea 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 IPD079Ea (*ipd079Ea*), PAT (*mo-pat*), and PMI (*pmi*) were introduced into corn line DP915635 via a two-step transformation process. Molecular analyses indicate that a single copy of each of the linked *ipd079Ea*, *mo-pat*, and *pmi* cassettes is present at a

single insertion site in the DP915635 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

Newly expressed proteins

IPD079Ea, PAT and PMI are newly expressed proteins present in DP915635. All three proteins were expressed throughout the plant, including at a low level in grain. Bioinformatic studies confirmed a lack of any significant amino acid sequence similarity between IPD079Ea and known protein toxins or allergens. Laboratory studies demonstrated that the IPD079Ea protein is susceptible to the action of digestive enzymes and would be thoroughly degraded before being absorbed during passage through the gastrointestinal tract. IPD079Ea is also susceptible to heat inactivation at the high temperatures typically used in food processing. 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 IPD079Ea, 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 DP915635 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 DP915635. Statistically significant differences were found between grain from DP915635 and the non-GM control for 4 of the 63 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 DP915635 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 DP915635. On the basis of the data provided in the present application and other available information, food derived from DP915635 is considered to be as safe for human consumption as food derived from non-GM corn cultivars.

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List of Abbreviations

Abbreviation	Description
ADF	acid detergent fibre
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
CI	confidence interval
COMPARE	COMprehensive Protein Allergen REsource
CRISPR	clustered regularly interspaced short palindromic repeats
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
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
ORF	open reading frame
PCR	polymerase chain reaction
SbS	Southern-by-sequencing
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TDF	total dietary fibre
µg	microgram
USDA	United States Department of Agriculture
WCR	western corn rootworm

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 DP915635, with the OECD Unique Identifier DP-915635-4. This corn line is tolerant to the herbicide glufosinate and protected against coleopteran insect pests, primarily western corn rootworm (WCR; *Diabrotica virgifera virgifera*).

Protection against corn rootworm is conferred by the expression of the *ipd079Ea* gene from the fern *Ophioglossum pendulum*, which encodes the IPD079Ea protein. Tolerance to the herbicide glufosinate is achieved by the expression of the maize-optimised *mo-pat* gene, derived from the bacterium *Streptomyces viridochromogenes*, encoding the enzyme phosphinothricin acetyltransferase (PAT). DP915635 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 IPD079Ea protein.

If approved, food derived from DP915635 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 PHR03 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). Canada produced 14.5 MT in 2022/23 (USDA 2023). Of the corn grown in the United States and Canada, an estimated 92% and ~90%, respectively, is GM.[3,4,5]

Corn is not a major crop in Australia or New Zealand – in 2021 these amounted to 0.306 and 0.209 MT respectively (FAOSTAT 2022). No GM corn is currently grown commercially in Australia or New Zealand.

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 2022).

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[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

[4] USDA Grain Report, CA14062, 2014: https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agricultural%20Biotechnology%20Annual_Ottawa_Canada_7-14-2014

[5] Statistics Canada, 2023: https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210004201

Corn has a long history of safe consumption as food by humans.[6] 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 Ophioglossum pendulum

The *ipd079Ea* gene is derived from the fern *Ophioglossum pendulum*, also known as Old World adders-tongue fern (Carlson et al 2022). While there are limited anecdotal accounts of *O. pendulum* having been used for food applications, the applicant notes there are no reports in the literature that it is poisonous to either humans or livestock.

2.2.2 Streptomyces viridochromogenes

The source of the *mo-pat* gene is the bacterium *Streptomyces 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 multiple food-producing crops for almost three decades (CERA 2011).

2.2.3 Escherichia coli

The *pmi* gene is derived from the bacterial species *Escherichia 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 DP915635. These genetic elements are non-coding sequences and are used to regulate the expression of *ipd079Ea*, *mo-pat* and *pmi*.

[6] A large proportion of corn produced is also used as animal feed.

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.

Details of some of the specific genetic elements and plasmids used in the construction of DP915635, 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, using a total of five plasmids, were used to construct corn line DP915635. The purpose of each of the five plasmids is summarised in Figure 1. The transformation methodology is outlined in the flowchart in Appendix 1 and summarised below.

In the first transformation step, microprojectile bombardment with four plasmids (see Figure 1) was used to insert a “landing pad” sequence from plasmid PHP73878 into the genome of corn line PHR03 using a clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) targeted insertion process. The helper plasmid PHP70605 was used to express the *zm*-45CR1 guide RNA and Cas9 endonuclease. The *zm*-45CR1 guide RNA directed Cas9 to create a double-stranded break between the endogenous *zm*-SEQ158 and *zm*-SEQ159 sequences in the PHR03 genome. The landing pad sequence in PHP73878 is flanked by identical *zm*-SEQ158 and *zm*-SEQ159 sequences, and was incorporated into the genome by homology-directed repair (HDR). This transformation step also utilised the helper plasmids PHP21139 and PHP21875 (Figure 1), which transiently expressed the corn-derived WUS and ODP2 proteins, respectively, for improved regeneration of corn plants following transformation (Lowe et al. 2016). No elements from any of the helper plasmids were incorporated into the corn genome.

The second transformation step used the trait plasmid PHP83175 (Figure 1; Appendix 2) and was *Agrobacterium*-mediated. Site-specific integration (SSI) of PHP83175-derived DNA into the landing pad introduced the *pmi*, *mo-pat* and *ipd079Ea* gene cassettes. Three additional gene cassettes (*zm-wus2, zm-odp2,* and *mo-Flp*) were also present in PHP83175 (see Appendix 3), but these cassettes were transiently expressed during transformation and were not integrated into the genome of DP915635. The WUS and ODP2 proteins performed the same function as in the first transformation step, and FLP recombinase allowed the SSI (see section 3.2). Selection using the PMI selectable marker, by growing transformed plant cells on media containing mannose, was only applicable following the second transformation step.

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 the evaluation of trait efficacy and agronomic performance, corn line DP915635 was selected.

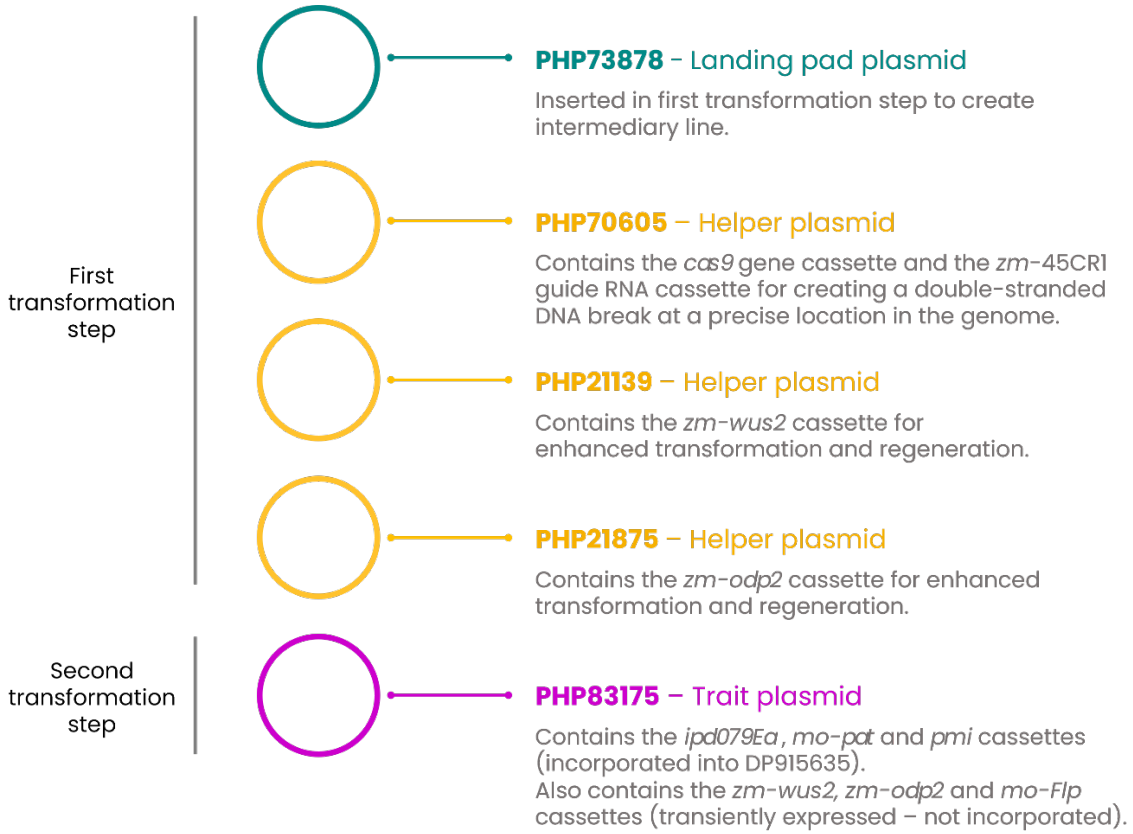

Figure 1. *Schematic of the five plasmids used in the two transformation steps to construct DP915635.*

3.2 Detailed description of inserted DNA

The sequential two-step transformation process used to create DP915635 resulted in a final DNA insert which contains sequences from two distinct plasmids: the landing pad plasmid PHP73878 and the trait plasmid PHP83175 (Figure 2).

The landing pad sequence, between *zm*-SEQ158 and *zm*-SEQ159 of the PHP73878 plasmid, was incorporated into the genome of the intermediary line. The landing pad sequence included the flippase recombinase target sites FRT1 and FRT6, which are homologous to the FRT1 and FRT6 sites present in the trait plasmid PHP83175. These sites allowed the exchange of the portion of the landing pad between FRT1 and FRT6 with the trait expression cassettes from the PHP83175 plasmid (Figure 2).

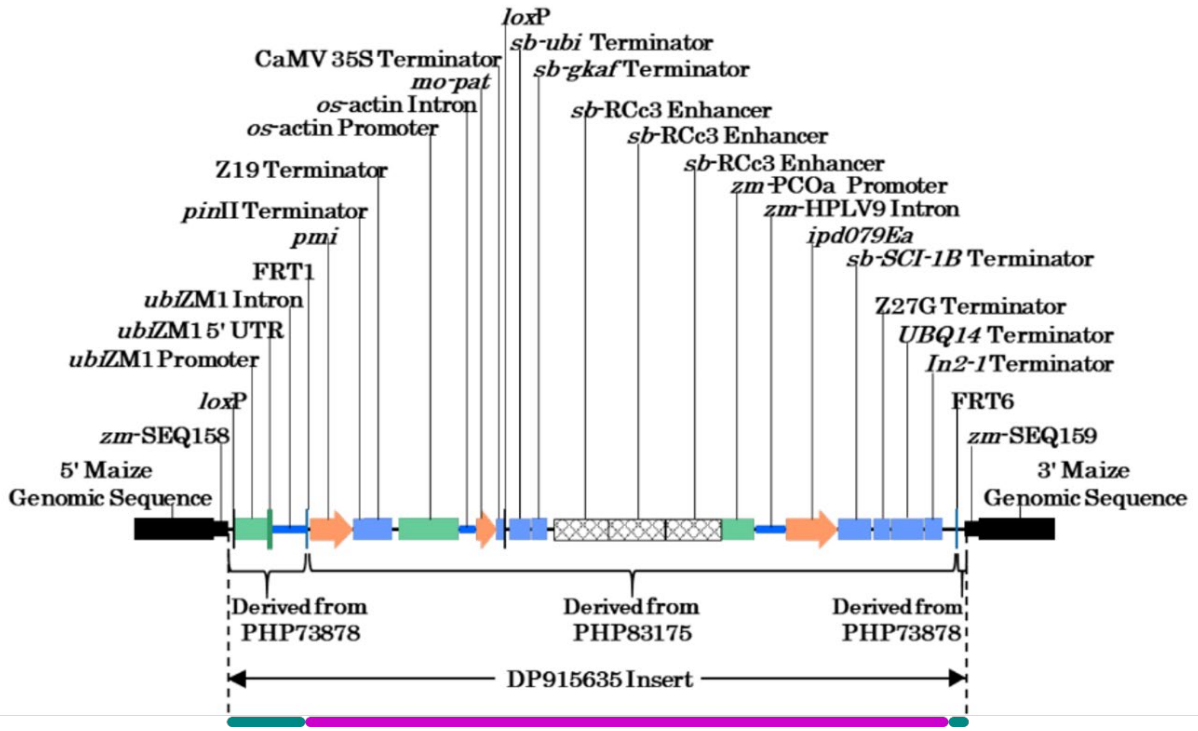

Figure 2. *Schematic of the inserted DNA in DP915635, derived from the landing pad plasmid PHP73878 (teal) and the trait plasmid PHP83175 (purple). Within the insert, green bars: promoters; orange arrows: coding sequences; thin blue bars: introns; thick blue bars: terminators; cross-hatched bars: enhancers.*

The expression cassettes from PHP83175 inserted into DP915635 are summarised in Table 1. 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 4.

Table 1: Expression cassettes contained in the DP915635 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 *ipd079Ea* cassettes: the terminators from the ubiquitin (*sb-ubi*) and γ-kafirin (*sb-gkaf*) genes from *Sorghum bicolor* (sorghum)	Confers tolerance to glufosinate
***ipd079Ea* cassette**	Three copies of the enhancer region from the root cortical RCc3 (*sb*-RCc3) gene from *Sorghum bicolor* (sorghum) Promoter region upstream of a PCO118362 mRNA sequence (*zm*-PCOa) from *Zea mays* (corn) Intron region from a predicted calmodulin 5 gene from *Zea mays* (*zm*-HPLV9)	*ipd079Ea* gene from *Ophioglossum pendulum*	Terminator from the subtilisin-chymotrypsin inhibitor *1B* (*sb-SCI-1B*) gene from *Sorghum bicolor* (sorghum) Three additional terminators are present: the terminators from the 27-kDa gamma zein (Z27G) gene from the *Zea mays* W64 line; the ubiquitin 14 (*UBQ14*) gene from *Arabidopsis thaliana*; and the *In2-1* gene from *Zea mays*	Confers resistance to corn rootworm

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 DP915635
- ensuring that the DP915635 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: DP915635 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	PHR03
Sanger sequencing	Section 3.4.3	T4	PHR03
Genetic stability	Section 3.4.4.1	T1, T2, T3, T4, T5	PHR03
Mendelian inheritance; expression of phenotype over multiple generations	Section 3.4.4.2	F1, T2, T3, T4, T5	N/A
Expression analysis of novel proteins	Sections 4.1.1.2, 4.1.2.1, 4.1.3.1	F1	PH1KTF/PHR03
Compositional analysis	Section 5	F1	PH1KTF/PHR03

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

A range of analyses were undertaken to characterise the genetic modification in DP915635. 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).

3.4.1 Number of integration site(s)

Leaf-derived genomic DNA from ten plants from the T1 generation of DP915635, along with DNA from a plant from the non-GM near-isogenic line PHR03 corn as a control, was analysed by SbS. The ten DP915635 plants consisted of 5 transgenic and 5 null segregant plants. Additionally, positive control samples were generated using the PHR03 genomic DNA spiked with either the PHP83175 trait plasmid, the PHP73878 landing pad plasmid and the PHP70605, PHP21139, and PHP21875 helper plasmids. 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. SbS analysis of each of the 5 transgenic 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, with the expected organisation, was integrated into the genome of DP915635.

The control contained sequence coverage above the background level (35x). However, these were due to the capture and sequencing of endogenous sequences from corn that were present in the inserted DNA. No junctions between plasmid DNA and genomic DNA were identified in the control or in the 5 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 five plasmids used in the development of DP915635 (Figure 1). Alignment of NGS reads from the controls or DP915635 to all five plasmid sequences confirmed there was no integration of backbone sequences into DP915635, including any antibiotic resistance genes.

3.4.3 Insert integrity and site of integration

The SbS analysis indicated that DP915635 contains a single copy of the intended insertion, with the expected organisation, and no unintended sequences or rearrangements. There was a single nucleotide A to C change identified in the *ubi*ZM1 promoter of the landing pad sequence (which regulates expression of the *pmi* cassette) which was present in all 5 DP915635 transgenic plants containing the insertion, suggesting it was derived from the initially transformed plant. An additional single nucleotide change was identified in the *os-actin* promoter of one plant, suggesting that this change arose spontaneously during the breeding process. PCR and sequencing analysis of the insert and flanking corn genomic regions was consistent with the SbS data and confirmed that the organisation of the insert in DP915635 is as expected, with the exception of the single nucleotide change in the *ubi*ZM1 promoter.

3.4.4 Stability of the genetic changes in corn line DP915635

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 *ipd079Ea* gene cassettes in DP915635. Leaf-derived genomic DNA from five generations of DP915635 (T1 – T5) was extracted, digested with the *Pvu* II restriction enzyme, and hybridised with labelled probes specific for the *pmi*, *mo-pat* and *ipd079Ea* gene cassettes. Genomic DNA from the non-GM corn line PHR03 served as a negative control, and PHR03 DNA spiked with plasmid PHP83175 served as a positive control to confirm probe hybridisation.

Hybridisation of each probe to the digested genomic DNA from DP915635 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 DP915635.

3.4.4.2 Phenotypic stability

Expression of phenotype over several generations

The inheritance pattern was assessed in five generations of DP915635 (F1, T2, T3, T4, and T5), using 100 plants per generation. Plants from each generation were evaluated by both quantitative and qualitative polymerase chain reaction (PCR) assays, using primers targeting the *pmi*, *mo-pat* and *ipd079Ea* genes, as well as other genetic elements associated with the insertion site. Plants were also examined phenotypically using a herbicide injury evaluation. Each plant was assessed visually for glufosinate-tolerance four to six 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 DP915635. Since the inserted DNA resides at a single locus within the DP915635 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, were 1:1 for the F1 generation, 3:1 for the T2 and T3 generations, and homozygous positive for the T4 and T5 generations. The results demonstrated the expected segregation ratio for each generation (Table 3). These results were compared to the results from the phenotypic analysis and the co-segregation of genotype and phenotype was confirmed, 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 DP915635 and is inherited predictably according to Mendelian principles in subsequent generations.

Table 3: Segregation results in five generations of DP915635

Generation	Expected segregation ratio (positive:negative)	Observed number of plants			Statistical analysis	
		Positive	Negative	Total	χ^2	p-value
T3	3:1	74	26	100	0.05	0.8174
F1	1:1	47	53	100	0.36	0.5485
T2	3:1	79	21	100	0.85	0.3556
T4	Homozygous positive	100	0	100	-	-
T5	Homozygous positive	100	0	100	-	-

3.4.5 Open reading frame analysis

A bioinformatic analysis of the DP915635 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel open reading frames (ORFs) had been created in DP915635 as a result of the DNA insertion, and whether any putative peptides encoded by the identified ORFs have the potential for allergenicity or toxicity.

All sequences of ≥ 30 amino acids (aa) in length spanning the 5' and 3' insert-flank junctions of DP915635, or contained within the insert itself, were translated *in silico* from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames. A total of 92 ORFs ≥ 30 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 ORFs or

putative peptides would be expressed *in planta*.

3.4.5.1 Bioinformatic analysis for potential allergenicity

The amino acid sequences in the ORFs 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 2020), there were 2,248 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 0.0001. 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.

No matches between the 92 putative peptides and proteins from the COMPARE database were identified in the FASTA alignment. One ORF – from the translated PMI protein sequence – produced an 8 contiguous amino acid match (DLSDKETT) to an allergen in the COMPARE database: a putative alpha-parvalbumin from frog (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 ORFs generated in DP915635 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 2020). 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 with a BLOSUM62 scoring matrix and an E-value threshold of 0.0001 was used.

No alignments were found between the 92 putative peptides and any known protein toxins. The novel ORFs in DP915635 therefore do not present a toxicity concern.

3.5 Conclusion

Corn line DP915635 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 *ipd079Ea* 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 DP915635. Bioinformatics analyses of the new ORFs ≥ 30 aa created by the modification did not raise any allergenicity or toxicity concerns.

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

[8] A564, A580, A1001, A1038, A1060, A1202, A1070

4 Characterisation and safety assessment of novel substances

4.1 Novel Proteins

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 substances are expressed in DP915635: the IPD079Ea insecticidal protein, which provides protection against corn rootworm 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. 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.

4.1.1 IPD079Ea

It has long been observed that insects consume ferns and mosses at a significantly lower rate compared to flowering plants (Hendrix 1980). More recently, ferns have been revealed as a promising source of substances with insecticidal activity, including proteins (Markham et al. 2006; Shukla et al. 2016; Simmons and Herman 2023). IPD079Ea, isolated from the fern species *Ophioglossum pendulum*, is a 479 amino acid, ~52 kDa protein encoded by the *ipd079Ea* gene. It contains a Membrane Attack Complex/Perforin and Cholesterol-Dependent Cytolysin (MACPF/CDC) domain (Carlson et al. 2022). The MACPF/CDC superfamily is distributed across all kingdoms of life, and operates via a pore-forming mechanism of action (Rosado et al. 2008), In plants, these proteins play a role in development and defence (Yu et al. 2020).

When expressed in corn, IPD079Ea provides protection against coleopteran insect species with a relatively narrow spectrum of activity, demonstrating the highest activity against WCR (Boeckman et al. 2022). IPD079Ea disrupts the midgut epithelial cells of corn rootworm larvae via a pore-forming mode of action similar to crystal (Cry) proteins from *Bacillus thuringiensis*, but likely achieves this by binding to a distinct set of midgut receptors (Carlson et al. 2022). A safety assessment of the IPD079Ea protein has recently been published (Carlson et al. 2022).

4.1.1.1 Characterisation of IPD079Ea expressed in DP915635 and equivalence to a bacterially-produced form

Plant-derived IPD079Ea protein was purified from DP915635 leaf tissue using immunoaffinity chromatography. The purified IPD079Ea fractions were combined and subsequently concentrated. To obtain sufficient quantities of IPD079Ea for use in safety studies, IPD079Ea was also expressed in *E. coli*, fused to an N-terminal His-tag. The *E. coli*-derived IPD079Ea

protein was purified using nickel affinity chromatography. After purification, the His-tag was cleaved using thrombin, and the tag and thrombin were then removed by heparin Sepharose chromatography.

The equivalence of the DP915635- and *E.coli*-derived IPD079Ea proteins must be established before the safety data and conclusions generated using *E. coli*-derived IPD079Ea can be applied to DP915635-derived IPD079Ea. In order to confirm the identity and equivalence of the DP915635- and *E. coli* derived IPD079Ea, a series of analytical tests were done, the results of which are summarised below.

Molecular weight. Samples of purified DP915635- and *E. coli*-derived IPD079Ea were subjected to SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. Both proteins migrated with an apparent molecular weight of approximately 52 kDa, as expected.

Immunoreactivity. Western blot analysis with an IPD079Ea-specific antibody showed that the protein being expressed in DP915635 and *E. coli* was indeed IPD079Ea, and that they have equivalent immunoreactivity.

Peptide mapping. DP915635-derived and *E. coli*-derived IPD079Ea were digested with trypsin or chymotrypsin and analysed by LC-MS/MS. For DP915635-derived IPD079Ea, the combined tryptic and chymotryptic peptides, covered 94.8% of the expected protein sequence (453 of 478 amino acids). For *E. coli*-derived IPD079Ea, the peptide coverage was 96% of the expected protein sequence (463 of 481 amino acids[9]).

N-terminal sequencing. Edman sequencing of the *E.coli*-derived IPD079Ea identified an N-terminal sequence which matched the first 10 residues of the expected sequence. The N-terminus of the DP915635-derived IPD079Ea was modified such that Edman sequencing was not possible, but peptide mapping of the protein identified that the N-terminal peptide matched the expected sequence based on the *ipd079Ea* gene sequence, and that the N-terminal methionine was absent, as expected (Bradshaw et al. 1998).

Glycosylation analysis. SDS-PAGE combined with a colourimetric glycoprotein detection procedure showed that the IPD079Ea proteins from both DP915635 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 IPD079Ea was evaluated in a 7-day insect bioassay. In this assay, WCR larvae were fed either a diet containing 50 ng IPD079Ea protein per mg diet wet weight or control diet containing ultrapure water. Larvae fed a diet containing IPD079Ea showed a mortality of 100%, compared to 13% in the control diet. This result demonstrates that *E. coli*-derived IPD079Ea protein is functionally active against WCR.

The results outlined in this section demonstrated that *E.coli*-derived IPD079Ea is structurally and biochemically equivalent to DP915635-derived IPD079Ea. The biological activity of *E. coli*-derived IPD079Ea was demonstrated in an insect bioassay and based on the structural and biochemical equivalence to DP915635-derived IPD079Ea, the two proteins are expected to be functionally equivalent. It can be concluded that *E. coli*-derived IPD079Ea is a suitable surrogate for use in the safety studies described in Section 4.1.1.3.

[9] Note that the extra 3 amino acids in the expected sequence of the *E. coli*-derived IPD079Ea are due to the presence of the N-terminal methionine, which is absent in the plant-derived protein, as well as an additional glycine and serine residue present as remnants of the His-tag (following thrombin cleavage).

4.1.1.2 Expression of IPD079Ea in DP915635 tissue

For analysis of the expression levels of IPD079Ea protein in DP915635, tissues were collected from six field-trial sites in representative corn-producing regions of the United States and Canada during the 2019 growing season.[10] Tissues were collected at varying stages of growth (see Figure 3 for a summary of corn growth stages). Tissues were lyophilised, homogenised (except pollen samples) and stored frozen until analysis.

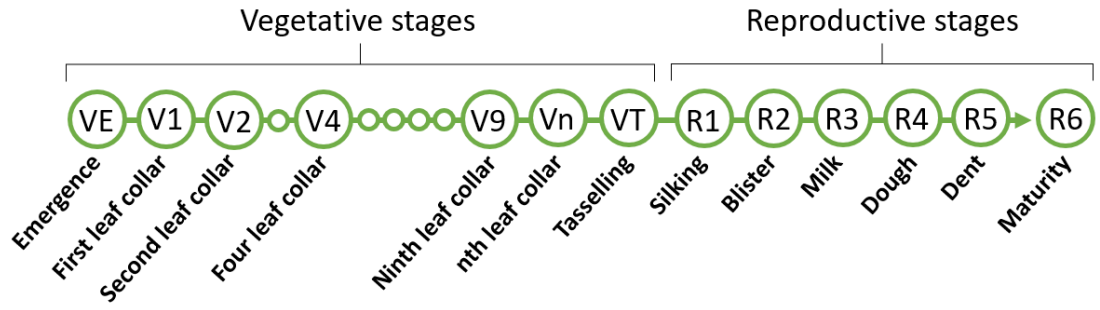

Figure 3. *Stages of corn growth. Grain is harvested at maturity (R6).*

Table 4: IPD079Ea concentrations (ng/mg dw[1]) in DP915635 tissues

Tissue (Growth Stage[2])	Mean	Range	Standard Deviation	LLOQ[3]
DP915635				
Root (V6)	16	9.9 – 26	4.2	0.069
Root (V9)	9.2	0.72 – 30	5.8	0.069
Root (R4)	1.2	0.28 – 2.7	0.66	0.069
Leaf (V9)	0.83	0.33 – 1.6	0.31	0.14
Leaf (R1)*	0.16	<0.14 – 0.29	0.11	0.14
Leaf (R4)	<0.14	<0.14	ND[4]	0.14
Pollen (R1)	0.95	0.58 – 1.3	0.18	0.28
Forage (R4)	0.25	0.086 – 0.46	0.088	0.046
Grain (R6)	0.18	0.075 – 0.36	0.065	0.069
Herbicide-Treated DP915635				
Root (V6)	18	5.7 – 25	4.1	0.069
Root (V9)	10	0.63 – 33	6.5	0.069
Root (R4)	1.1	0.36 – 2.0	0.52	0.069
Leaf (V9)	0.82	0.45 – 2.6	0.46	0.14
Leaf (R1)*	0.080*	<0.14 – 0.16	0.027	0.14
Leaf (R4)	<0.14	<0.14	ND[4]	0.14
Pollen (R1)	0.88	0.62 – 1.3	0.20	0.28
Forage (R4)	0.24	0.12 – 0.40	0.072	0.046
Grain (R6)*	0.16	<0.069 – 0.30	0.068	0.069

1. dw - dry weight 2. Growth Stage abbreviations – see Figure 3 3. LLOQ – lower limit of quantitation 4. ND – not determined
* For these tissues, some, but not all, samples, gave results below the LLOQ. A value of half the LLOQ was assigned to these samples to calculate the mean and standard deviation.

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

Expression levels of IPD079Ea were quantified in each tissue using a quantitative enzyme-linked immunosorbent assay (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 DP915635 were collected.

Results from the ELISA (Table 4) showed IPD079Ea expression in herbicide-treated DP915635 was highest on a dry weight (dw) basis in root tissue, which is the target tissue for WCR consumption. The lowest level of IPD079Ea expression in herbicide-treated DP915635 was in leaf tissue at the R4 growth stage, which was below the lower limit of quantitation (LLOQ) for the assay. IPD079Ea was also detected at a very low level in the grain. Similar levels of IPD079Ea were detected in DP915635 not treated with glufosinate.

4.1.1.3 Safety of the introduced IPD079Ea

Data were provided to assess the potential toxicity and allergenicity of IPD079Ea.

Bioinformatic analyses of IPD079Ea

In silico analyses, using the same criteria described in section 3.4.5.1, were performed to compare the IPD079Ea amino acid sequence to known allergenic proteins in the COMPARE database (January 2020). The search did not identify any known allergens with homology to IPD079Ea. 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 IPD079Ea sequence and proteins in the allergen database.

To assess the similarity of IPD079Ea to known toxins, the applicant provided the results of *in silico* analyses comparing the IPD079Ea amino acid sequence to proteins identified as "toxins" in the same in-house database described in section 3.4.5.2 (January 2020). 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 IPD079Ea.

Susceptibility of IPD079Ea to digestion with pepsin and pancreatin

E. coli-derived IPD079Ea (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, 30, and 60 min. Controls included bovine serum albumin (BSA) or β-lactoglobulin proteins in SGF incubated for 0, 1, and 60 min, no protein in SGF incubated for 60 min, and IPD079Ea in water or a gastric control solution that did not contain pepsin and was 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 IPD079Ea remaining in the reaction mix. Some low molecular weight bands were evident in the IPD079Ea samples 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. IPD079Ea remained intact after 60 min in water and in the gastric solution without pepsin. These results indicate that IPD079Ea is rapidly digested by pepsin.

E. coli-derived IPD079Ea protein was also incubated with pancreatin[11] (20 µg enzyme/µg protein) at 37°C over a 0-60 min time course, in a simulated intestinal fluid (SIF) system at a neutral pH of ~7.5. Controls included BSA or β-lactoglobulin proteins in SIF incubated for 0

[11] Pancreatin is a mixture of proteolytic enzymes

and 60 min, no protein in SIF incubated for 60 min, and IPD079Aa in water or an intestinal control solution that did not contain pancreatin and was 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 IPD079Ea remained in the reaction mix throughout the 60 min incubation period, though the band corresponding to intact IPD079Ea did gradually decrease in intensity. Smaller molecular weight fragments were also evident throughout the digestion period, some of which increased in intensity during the digestion period. The BSA control remained undigested after 60 min in SIF and the β-lactoglobulin control was digested within 1 min in SIF. IPD079Ea remained intact after 60 min in water and in the intestinal solution without pancreatin. These results indicated that IPD079Ea is gradually digested by pancreatin.

IPD079Ea was also subjected to a sequential digestion with pepsin (SGF, as above) followed by pancreatin (SIF, as above). IPD079Ea was first incubated with SGF for 10 min, then with SIF over a 0-60 min time course. The low molecular weight bands 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 IPD079Ea would be fully degraded by gastric and intestinal enzymes in the human digestive system.

Bioactivity of IPD079Ea after exposure to heat

The thermal stability of IPD079Ea was evaluated by assessing the functional activity of the heat-treated IPD079Ea protein in a 7-day insect bioassay. *E.coli*-derived IPD079Ea was incubated for 30-35 minutes at 25°C, 50°C, 75°C, or 95°C before incorporation into an artificial diet for WCR larvae. Control diets contained either ultrapure water, or unheated IPD079Ea. The test diets and the unheated control diet contained a target concentration of 50 ng IPD079Ea protein per mg diet wet weight. Each diet was provided to 30 individual WCR larvae (except for the unheated control diet which was provided to 24 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. ELISA analysis confirmed the dose and homogeneity of the IDP079Ea protein during the assay.

Table 5: Bioactivity of heat-treated IPD079Ea in a diet fed to insect larvae

Treatment description	Incubation condition	Number of observations[1]	Total number of surviving organisms	Mortality (%)	p-value[2]	Weight of surviving organisms (mg)
Water diet	-	29	28	3.45	-	0.486 ± 0.130
Unheated control diet	-	21	1	95.2	-	0.00
Test diet	25°C	28	1	96.4	0.8214	0.100
	50°C	28	27	3.57	<0.0001	0.448 ± 0.101
	75°C	29	28	3.45	<0.0001	0.489 ± 0.131
	95°C	30	30	0	<0.0001	0.447 ± 0.114

1. All treatments began with 30 organisms, except the unheated control which began with 24 organisms. Organisms were excluded from the final tally if they were missing, or if assay wells contained more than one organism. 2. 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.

The results demonstrated that when heated to temperatures of 50-95°C, the ability of the IPD079Ea protein to cause WCR mortality was effectively abolished, with mortality rates of <4% for the larvae fed diets containing IPD079Ea heat-treated at 50°C, 75°C, or 95°C (Table 5). Fisher's exact test was used to determine whether the mortality rate of WCR that had

been fed diets containing the heated IPD079Ea was smaller than that of those fed the unheated control diet. For IPD079Ea heated to 50°C, 75°C, or 95°C, the decrease in activity against WCR larvae was statistically significant (p-value <0.05) compared to the unheated control. The IPD079Ea protein that was heat-treated at 25°C did not have a statistically significant decrease in activity compared to the unheated control (Table 5). These data indicate that IPD079Ea is heat labile at temperatures ≥ 50°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 IPD079Ea was submitted by the applicant as additional supporting information. FSANZ has had regard to the results of this study.

In summary, the IPD079Ea protein was administered to mice (6/sex/group) by oral gavage in a split dose totalling 5000 mg/kg bodyweight on test day 1. Water was used as the vehicle control. Animals were monitored over the 14 day test period for bodyweight, mortality and other clinical signs, and then subject to gross pathology following scheduled death on day 15. No treatment-related adverse effects were observed.

4.1.1.4 Conclusion

A range of characterisation studies were performed on plant-derived IPD079Ea confirming its identity, structure and biochemistry as well as equivalence of the corresponding protein derived from a bacterial expression system. The bacterially-expressed IPD079Ea was also shown to be functional. Expression of IPD079Ea in DP915635 was highest in root tissue and lowest in leaf and grain tissue. Bioinformatic analyses showed IPD079Ea did not share any meaningful homology with any known allergens or toxins. IPD079Ea was heat labile at ≥ 50°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 IPD079Ea protein is unlikely to be toxic or allergenic to humans.

4.1.2 PAT

The *mo-pat* gene in DP915635 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 *mo-pat* gene in DP915635 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 kilodaltons (kDa).

The PAT enzyme has been used to confer glufosinate-tolerance in crops for approximately 25 years (CERA 2011; CERA 2016). Since 2002, FSANZ has assessed and approved numerous events with *pat*-encoded glufosinate-tolerance. There have been no credible reports of adverse effects on human health since it was introduced into food.

4.1.2.1 Expression of PAT in DP915635 tissue

PAT expression was determined using an ELISA on the same processed tissue samples analysed for IPD079Ea (Section 4.1.1.2). Results from the ELISA (Table 6) showed PAT expression in herbicide-treated DP915635 was highest on a dry weight (dw) basis in pollen at the early reproductive stage (R1). The lowest level of PAT expression in herbicide-treated DP915635 was in root tissue at the R4 growth stage. PAT was also detected in the grain, though at a much lower level than in pollen. Similar levels of PAT were detected in DP915635 not treated with glufosinate.

Table 6: PAT concentrations (ng/mg dw[1]) in DP915635 tissues

Tissue (Growth Stage[2])	Mean	Range	Standard Deviation	LLOQ[3]
DP915635				
Root (V6)	14	6.3 – 23	3.7	0.054
Root (V9)	7.3	3.6 – 14	3.3	0.054
Root (R4)	1.4	0.57 – 2.7	0.61	0.054
Leaf (V9)	4.4	2.6 – 8.4	1.3	0.11
Leaf (R1)	7.5	6.6 – 9.0	1.1	0.11
Leaf (R4)	3.8	1.9 – 5.4	0.89	0.11
Pollen (R1)	80	47 – 110	13	0.22
Forage (R4)	9.3	3.6 – 14	2.5	0.036
Grain (R6)	6.4	3.9 – 9.9	1.5	0.054
Herbicide-Treated DP915635				
Root (V6)	13	6.6 – 17	2.6	0.054
Root (V9)	8.6	2.6 – 19	4.3	0.054
Root (R4)	1.7	0.54 – 3.3	0.66	0.054
Leaf (V9)	5.0	2.9 – 10	1.7	0.11
Leaf (R1)	7.0	5.5 – 9.6	1.0	0.11
Leaf (R4)	3.8	1.9 – 5.0	0.85	0.11
Pollen (R1)	79	64 – 98	10	0.22
Forage (R4)	9.2	4.0 – 16	2.3	0.036
Grain (R6)	7.3	1.6 – 13	2.9	0.054

1. dw - dry weight 2. Growth Stage abbreviations – see Figure 3 3. LLOQ – lower limit of quantitation

4.1.2.2 Safety of the introduced PAT

The PAT protein encoded by the *pat* or *mo-pat* gene has been considered in numerous previous FSANZ safety assessments, including 12 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 (ILSI 2016; Hammond et al. 2011; Delaney et al. 2008; Hérouet et al. 2005).

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 DP915635 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. The results do not alter conclusions reached in previous assessments.

4.1.2.3 Conclusion

The data provided by the applicant confirms the PAT expressed in DP915635 is identical to previously assessed PAT proteins. The protein is expressed in various plant tissues, including grain. Updated bioinformatic analyses confirm that PAT has no similarity with known allergens or toxins that is of significance or concern.

4.1.3 PMI

The *pmi* gene in DP915635 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 kilodaltons (kDa). PMI has been assessed by FSANZ previously as a novel protein in 6 corn lines and one rice line.

4.1.3.1 Expression of PMI in DP915635 tissue

PMI expression was determined using an ELISA on the same processed tissue samples analysed for IPD079Ea and PAT (Section 4.1.1.1). Results from the ELISA (Table 7) showed PMI expression in herbicide-treated DP915635 was highest on a dry weight (dw) basis in leaf tissue at the R4 growth stage. The lowest level of PMI expression in herbicide-treated DP915635 was in root tissue at the R4 growth stage. PMI was also detected in the grain, though at a much lower level than in pollen. Similar levels of PMI were detected in DP915635 not treated with glufosinate.

4.1.3.2 Safety of the introduced PMI

The PMI protein has been previously assessed by FSANZ in 6 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), and DP51291 (Application A1270; FSANZ 2023), as well as in rice line GR2E (Application A1038; FSANZ 2017). 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 DP915635 is identical in amino acid 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. The results do not alter conclusions reached in previous assessments.

4.1.3.3 Conclusion

The data presented by the applicant confirms the PMI expressed in DP915635 is identical to previously assessed PMI proteins. The protein is expressed in various plant tissues,

including grain. Updated bioinformatic analyses confirm that PMI has no similarity with known allergens or toxins that is of significance or concern.

Table 7: PMI concentrations (ng/mg dw[1]) in DP915635 tissues

Tissue (Growth Stage[2])	Mean	Range	Standard Deviation	LLOQ[3]
DP915635				
Root (V6)	6.1	3.6 – 9.6	1.5	0.27
Root (V9)	4.8	1.8 – 9.6	2.1	0.27
Root (R4)	2.3	1.2 – 4.2	0.69	0.27
Leaf (V9)	6.6	3.4 – 11	2.0	0.054
Leaf (R1)	13	12 – 14	1.1	0.054
Leaf (R4)	27	20 – 38	3.5	0.054
Pollen (R1)	23	17 – 26	2.8	1.1
Forage (R4)	8.2	5.4 – 11	1.6	1.8
Grain (R6)	3.1	1.5 – 5.7	1.1	0.27
Herbicide-treated DP915635				
Root (V6)	6.4	4.2 – 11	1.6	0.27
Root (V9)	5.1	2.1 – 8.4	1.8	0.27
Root (R4)	2.5	1.5 – 3.9	0.72	0.27
Leaf (V9)	6.6	3.8 – 11	2.0	0.54
Leaf (R1)	14	9.0 – 28	3.9	0.54
Leaf (R4)	28	20 – 34	3.1	0.54
Pollen (R1)	22	17 – 26	2.4	1.1
Forage (R4)	8.9	5.4 – 11	1.3	1.8
Grain (R6)	4.0	1.4 – 8.4	1.8	0.27

1. dw - dry weight 2. Growth Stage abbreviations – see Figure 3 3. LLOQ – lower limit of quantitation

4.2 Herbicide metabolites

FSANZ has assessed the novel herbicide metabolites for glufosinate in GM crops in multiple previous applications. These previous assessments indicate the spraying of DP915635 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 DP915635, further assessment is not required.

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

DP915635 corn (F1 generation), a non-GM control corn of similar genetic background (PH1KTF/PHR03), and a total of 20 non-GM commercial reference corn lines[12] were grown and harvested from eight field trial sites in the United States and Canada during the 2019 growing season.[13] 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 DP915635 corn, non-GM control corn, and four reference corn lines selected from the 20 non-GM reference lines. Plants were grown under agronomic field conditions typical for each growing region. A herbicide treatment of glufosinate was applied to DP915635.

At maturity (R6 growth stage), grain was harvested from all plots, with reference and control grain collected prior to glufosinate-treated DP915635 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.

[12] The twenty reference corn lines: 5513, P0506, 35A52, P0604, P0760, 5883, P0993, 5939, 5828, P1151, P1197, 6158, P0928, P1105, P1345, P1319, P1395, P1422, 33Y74, and 6575

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

A total of 70 analytes in grain were assessed (see Figure 4 for a complete list, not including moisture). For 7 of these analytes (listed in grey in Figure 4) all samples of both DP915635 and the non-GM control gave results below the assay lower limit of quantification (LLOQ) and were therefore not analysed statistically.

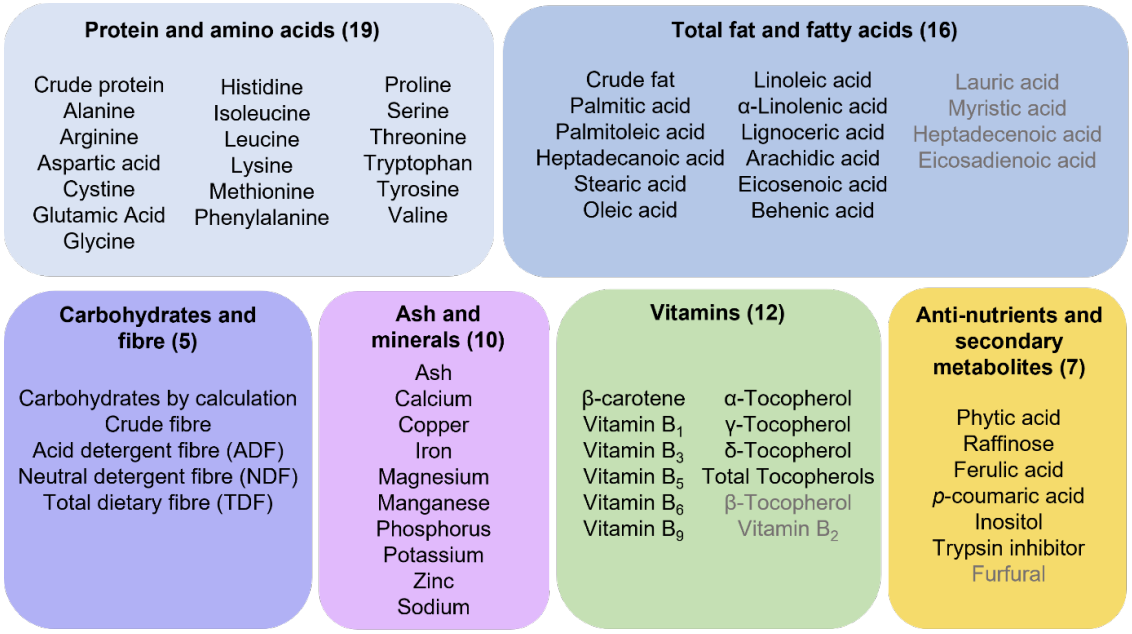

Figure 4. *Analytes measured in DP915635 grain samples. Analytes listed in grey text had all samples below the LLOQ and were excluded from statistical analysis. The analytes listed in black text, as well as moisture, were analysed fully.*

For the remaining 63 analytes, 'descriptive statistics' (mean, range and 95% confidence interval) were generated. For 60 of these analytes, where both DP915635 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 3 analytes (copper, δ-tocopherol and raffinose), >50% of either DP915635 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 DP915635 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. 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 DP915635 and the control were compared to tolerance intervals derived from an in-house database containing compositional analyses from 167 non-GM commercial corn lines cultivated across 171 unique environments in North and South America, from 2003-2018. Tolerance intervals are expected (with 95% confidence) to contain at least 99% of the values for corresponding analytes of the conventional maize population (Hong et al 2014). In addition, compositional data from the non-GM reference varieties grown concurrently in the same trial as DP915635 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 2019). 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 70 analytes measured in grain, mean values were provided for 63 analytes. Of these, statistically significant differences ($p < 0.05$) between herbicide-treated corn line DP915635 and the non-GM control were found for palmitoleic acid, lignoceric acid, iron, and *p*-coumaric acid. A summary of these 4 analytes is provided in Figure 5. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the Application dossier[14] (pages 110-123).

For the 4 analytes for which a statistically significant difference was found, all had FDR-adjusted p-values of >0.05, 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 DP915635 mean from the control mean was less than 7% (Figure 5a). As can be observed in Figure 5 (panels b-e), the DP915635 mean for each of these 4 analytes was within the control range value, indicating that DP915635 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. For all 4 analytes, the observed DP915635 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 5, 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 DP915635 when compared with conventional non-GM corn cultivars already available in agricultural markets. Grain from DP915635 can therefore be regarded as equivalent in composition to grain from conventional non-GM corn.

[14] https://www.foodstandards.gov.au/code/applications/Pages/A1272---Food-derived-from-herbicide-tolerant-and-insect-protected-corn-line-DP915635.aspx

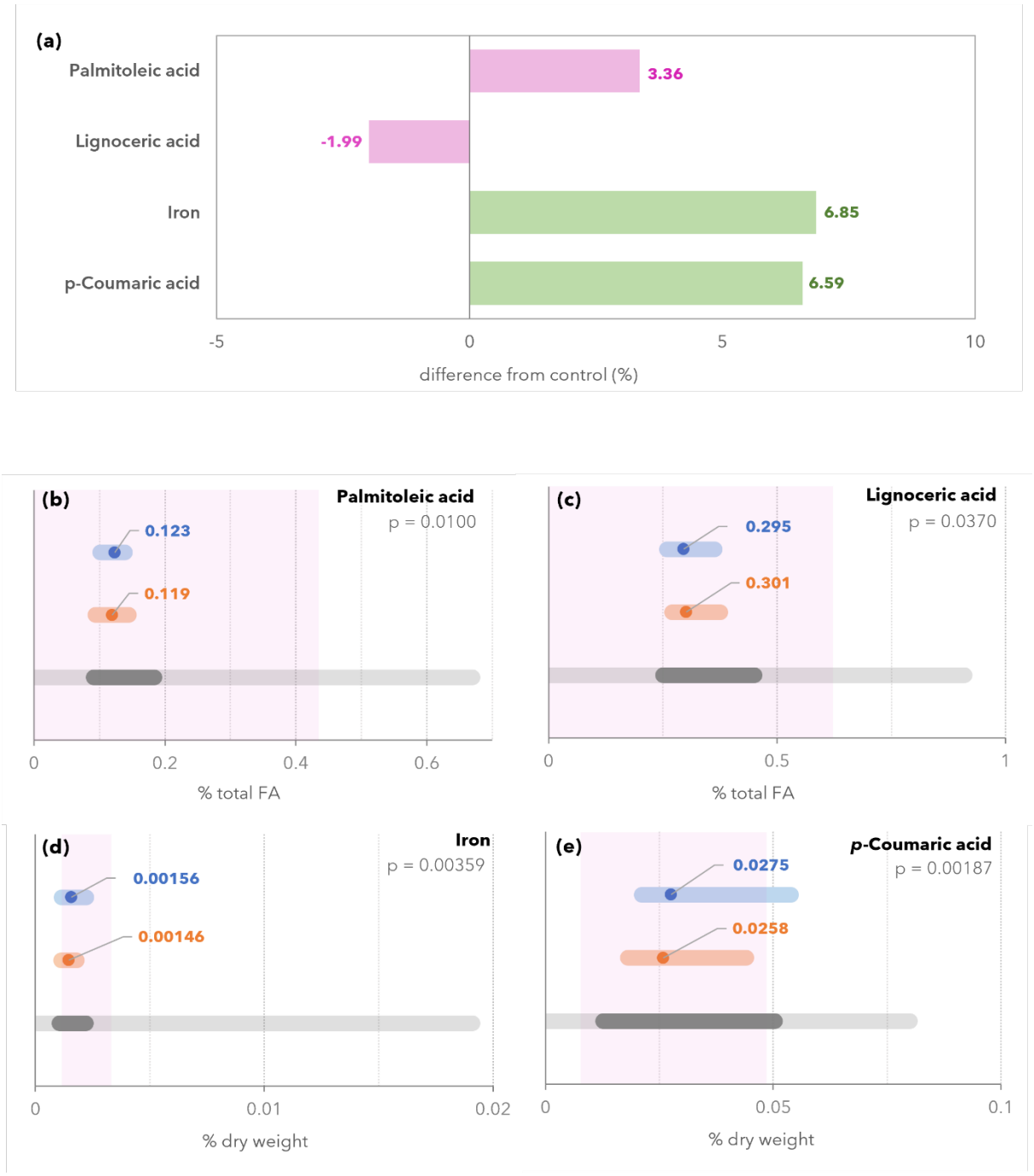

Figure 5. *Visual summary of statistically significant compositional differences between DP915635 and the conventional control corn.* **(a)** *Percentage deviation of the mean DP915635 value from the mean control value for each of the 4 analytes for which a statistically significant difference was found.* **(b) – (e)** *Measured means (dots) and ranges (coloured bars) for DP915635 (blue) and the conventional control (orange) for the 4 analytes as labelled. The light and dark grey bars represent the publicly-available range of values and in-study reference 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.

DP915635 is the result of genetic modifications to confer tolerance to the herbicide glufosinate and protection against corn rootworm 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 DP915635 compared with conventional non-GM corn cultivars. The introduction of food derived from DP915635 into the food supply is therefore expected to have negligible nutritional impact.

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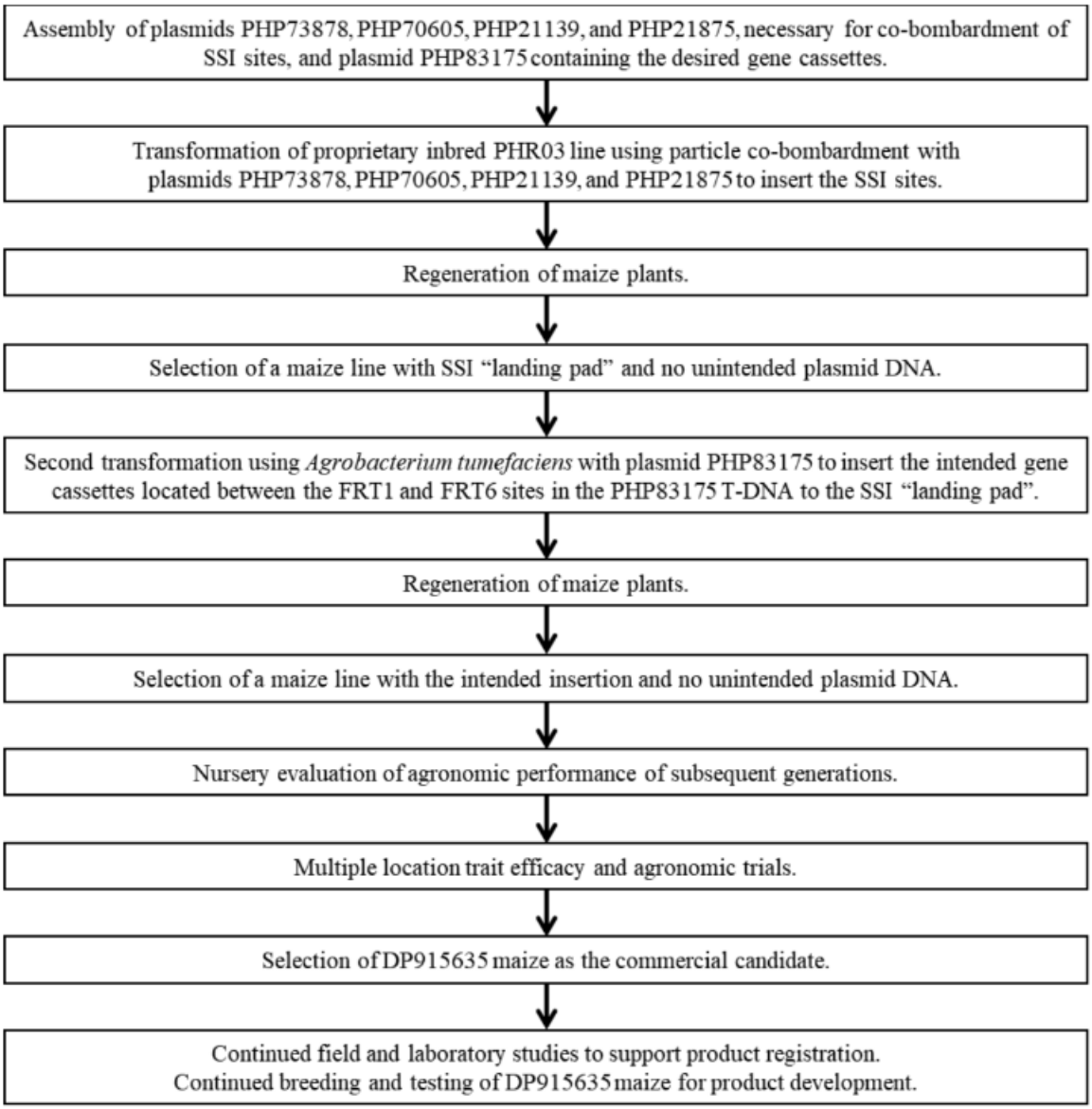
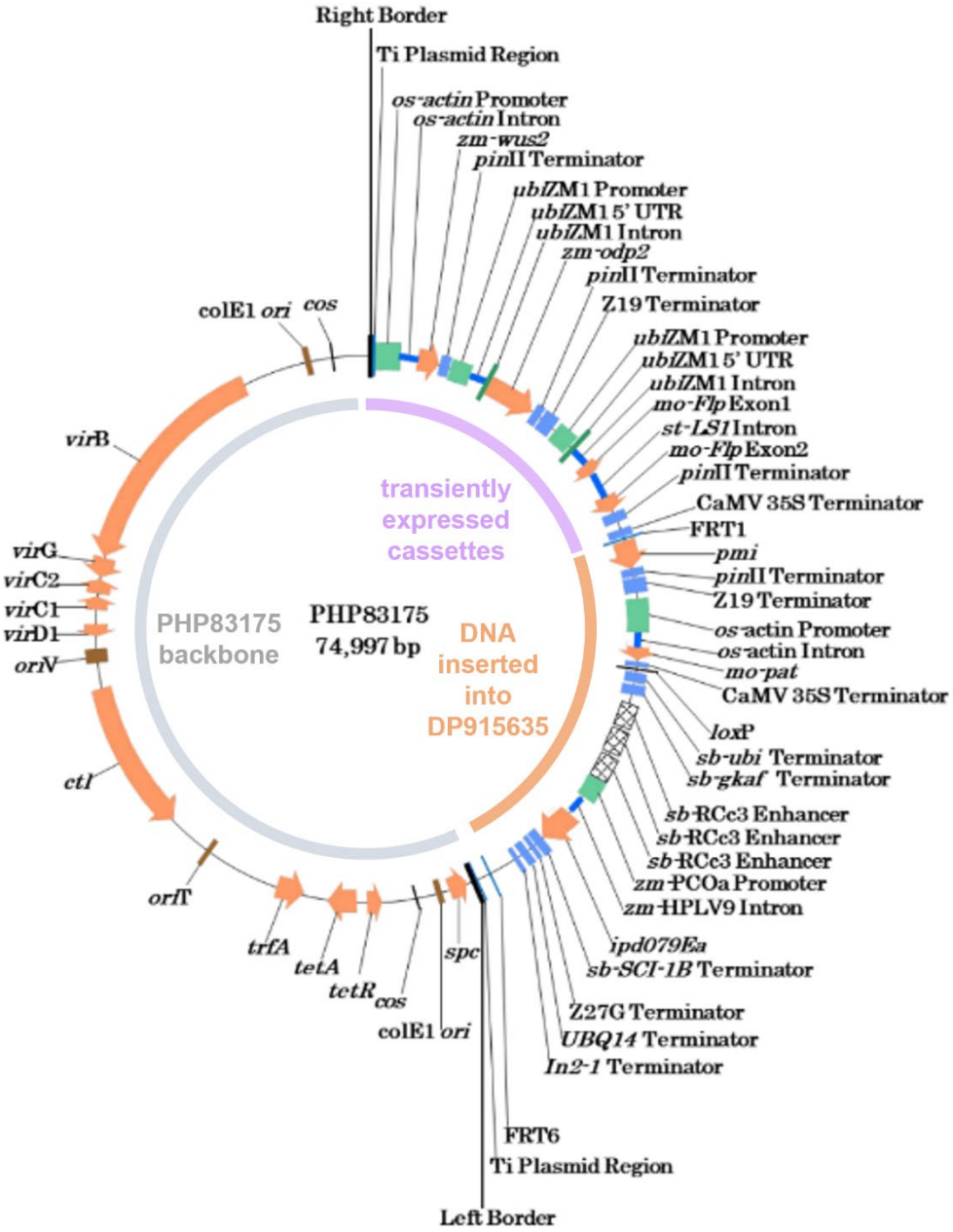
Appendix 1

Flowchart showing the development process used for creation of DP915635 corn



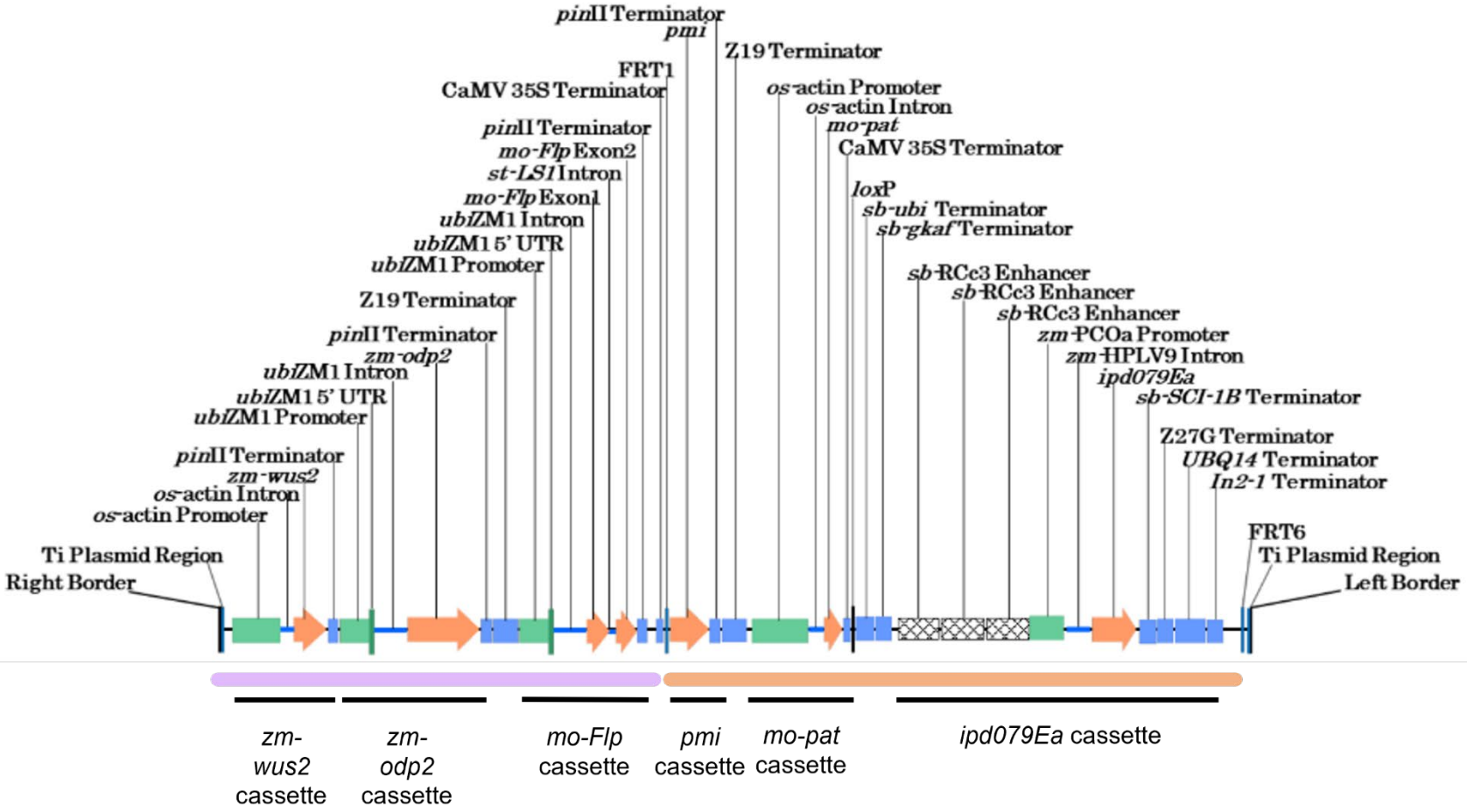
Appendix 2

PHP83175 plasmid map



Appendix 3

Schematic of the expression cassettes in plasmid PHP83175. The region that was integrated into DP915635 is highlighted in orange, and is bounded by FRT1 and FRT6 sites. The region highlighted in purple was not incorporated into the genome of DP915635.



Appendix 4

PHP83175-derived genetic elements

Genetic element	Relative position	Size (bp)	Description, Source & Reference
Right Border (RB)	1-25	25	T-DNA Right Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)
Ti Plasmid Region	26-75	50	Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)
Intervening Sequence	76-415	340	DNA sequence used for cloning (synthetic)
***zm-wus2* cassette**			
os-actin Promoter	416-2,097	1,682	Promoter region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159 ; GenBank accession EU155408.1)
os-actin Intron	2,098-2,566	469	Intron region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159 ; GenBank accession EU155408.1)
Intervening Sequence	2,567-2,571	5	DNA sequence used for cloning (synthetic)
zm-wus2	2,572-3,480	909	*Wuschel 2* gene from *Zea mays* (Lowe et al. 2016; Mayer et al. 1998)
Intervening Sequence	3,481-3,481	1	DNA sequence used for cloning (synthetic)
*pin*II Terminator	3,482-3,792	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al. 1989; Keil et al. 1986)
Intervening Sequence	3,793-3,862	70	DNA sequence used for cloning (synthetic)
***zm-odp2* cassette**			
*ubi*ZM1 Promoter	3,863-4,762	900	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 5′ UTR	4,763-4,845	83	5' untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 Intron	4,846-5,858	1,013	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
Intervening Sequence	5,859-5,876	18	DNA sequence used for cloning (synthetic)
zm-odp2	5,877-8,009	2,133	Ovule development protein 2 gene from *Zea mays* (GenBank accession XM008676474; US Patent 8420893)
Intervening Sequence	8,010-8,078	69	DNA sequence used for cloning (synthetic)
*pin*II Terminator	8,079-8,389	311	Terminator region from the Solanum tuberosum (potato) proteinase inhibitor II gene (An et al. 1989; Keil et al. 1986)
Intervening Sequence	8,390-8,405	16	DNA sequence used for cloning (synthetic)
Z19 Terminator	8,406-9,147	742	Terminator region from the *Zea mays* 19-kDa zein gene (GenBank accession KX247647; Dong et al. 2016)
Intervening Sequence	9,148-9,168	21	DNA sequence used for cloning (synthetic)

Genetic element	Relative position	Size (bp)	Description, Source & Reference
***mo-Flp* cassette**			
*ubi*ZM1 Promoter	9,169-10,068	900	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 5' UTR	10,069-10,151	83	5' untranslated region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 Intron	10,152-11,164	1,013	Intron region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
Intervening Sequence	11,165-11,194	30	DNA sequence used for cloning (synthetic)
mo-Flp Exon1	11,195 11,828	634	Maize-optimized exon 1 of the flippase gene from *Saccharomyces cerevisiae* (Dymecki 1996)
st-LS1 Intron	11,829-12,017	189	Intron region from the *Solanum tuberosum* (potato) *LS1* gene (Eckes et al. 1986)
mo-Flp Exon2 .	12,018-12,655	638	Maize-optimized exon 2 of the flippase gene from *Saccharomyces cerevisiae* (Dymecki 1996)
Intervening Sequence	12,656 -12,660	5	DNA sequence used for cloning (synthetic)
*pin*II Terminator	12,661-12,971	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II (An et al. 1989; Keil et al. 1986)
Intervening Sequence	12,972-13,025	54	DNA sequence used for cloning (synthetic)
*att*B4	13,026-13,046	21	Bacteriophage lambda integrase recombination site (Cheo et al. 2004)
Intervening Sequence	13,047-13,125	79	DNA sequence used for cloning (synthetic)
CaMV 35S Terminator	13,126-13,319	194	35S terminator region from the cauliflower mosaic virus genome (Franck et al. 1980; Guilley et al. 1982)
Intervening Sequence	13,320-13,334	15	DNA sequence used for cloning (synthetic)
FRT1	13,335-13,382	48	Flippase recombination target site from *Saccharomyces cerevisiae* (Proteau et al. 1986)
Intervening Sequence	13,383-13,400	18	DNA sequence used for cloning (synthetic)
***pmi* cassette**			
pmi	13,401-14,616	1,216	Phosphomannose isomerase gene from *Escherichia coli including* 5' and 3' untranslated regions (UTR) (Negrotto et al. 2000): 5' UTR at bp 13,401-13,404 (4 bp); Coding sequence at bp 13,405-14,580 (1,176 bp) 3' UTR at bp 14,581-14,616 (36 bp)
Intervening Sequence	14,617-14,626	10	DNA sequence used for cloning (synthetic)
*pin*II Terminator	14,627-14,937	311	Terminator region from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (An et al. 1989; Keil et al. 1986)
Intervening Sequence	14,938-14,947	10	DNA sequence used for cloning (synthetic)
Z19 Terminator	14,948-15,689	742	Terminator region from the *Zea mays* 19-kDa zein gene (GenBank accession KX247647; Dong et al. 2016)
Intervening Sequence	15,690-15,892	203	DNA sequence used for cloning (synthetic)

Genetic element	Relative position	Size (bp)	Description, Source & Reference
***mo-pat* cassette**			
os-actin Promoter	15,893-17,574	1,682	Promoter region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159; GenBank accession EU155408.1)
os-actin Intron	17,575-18,043	469	Intron region from the *Oryza sativa* (rice) actin gene (GenBank accession CP018159 ; GenBank accession EU155408.1)
Intervening Sequence	18,044-18,058	15	DNA sequence used for cloning (synthetic)
mo-pat	18,059-18,610	552	Maize-optimized phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes* (Wohlleben et al. 1988)
Intervening Sequence	18,611-18,628	18	DNA sequence used for cloning (synthetic)
CaMV 35S Terminator	18,629-18,822	194	355 terminator region from the cauliflower mosaic virus genome (Franck et al. 1980; Guilley et al. 1982)
Intervening Sequence	18,823-18,843	21	DNA sequence used for cloning (synthetic)
*lox*P	18,844-18,877	34	Bacteriophage P1 recombination site recognized by Cre recombinase (Dale and Ow 1990)
Intervening Sequence	18,878-18,973	96	DNA sequence used for cloning (synthetic)
sb-ubi Terminator	18,974-19,557	584	Terminator region from the *Sorghum bicolor* (sorghum) ubiquitin gene (Phytozome gene ID Sobic.004G049900.1; US Patent 9725731; Abbitt 2017)
Intervening Sequence	19,558-19,598	41	DNA sequence used for cloning (synthetic)
sb-gkaf Terminator	19,599-20,062	464	Terminator region from the *Sorghum bicolor* (sorghum) γ-kafirin gene (de Freitas et al. 1994)
Intervening Sequence	20,063-20,095	33	DNA sequence used for cloning (synthetic)
*att*B1	20,096 20,119	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway cloning system (Hartley et al. 2000; Katzen 2007)
Intervening Sequence	20,120-20,157	38	DNA sequence used for cloning (synthetic)
***ipd079Ea* cassette**			
sb-RCc3 Enhancer	20,158-21,738	1,581	Enhancer region, showing root-specific activity, from the *Sorghum bicolor* (sorghum) root cortical RCc3 (*sb*-RCC3) gene (WO Patent 2012/112411; Diehn and Peterson-Burch 2012)
Intervening Sequence	21,739-21,744	6	DNA sequence used for cloning (synthetic)
sb-RCc3 Enhancer	21,745-23,325	1,581	Enhancer region, showing root-specific activity, from the *Sorghum bicolor* (sorghum) root cortical RCc3 (*sb*-RCC3) gene (WO Patent 2012/112411; Diehn and Peterson-Burch 2012)
Intervening Sequence	23,326-23,338	13	DNA sequence used for cloning (synthetic)
sb-RCc3 Enhancer	23,339-24,922	1,584	Enhancer region, showing root-specific activity, from the *Sorghum bicolor* (sorghum) root cortical RCc3 (*sb*-RCC3) gene (WO Patent 2012/112411; Diehn and Peterson-Burch 2012)

Genetic element	Relative position	Size (bp)	Description, Source & Reference
zm-PCOa Promoter	24,923-25,833	911	Promoter region upstream of a *Zea mays* PCO118362 mRNA sequence identified as having root-specific activity (WO Patent 2017/222821; Crow et al. 2017)
Intervening Sequence	25,834-25,851	18	DNA sequence used for cloning (synthetic)
zm-HPLV9 Intron	25,852-26,707	856	Intron region from the *Zea mays* ortholog of an *Oryza sativa* (rice) hypothetical protein (*zm*-HPLV9) gene, a predicted *Zea mays* calmodulin 5 gene (Phytozome gene ID Zm00008a029682, WO Patent 2016109157 ; Abbitt and Shen 2016)
Intervening Sequence	26,708-26,716	9	DNA sequence used for cloning (synthetic)
ipd079Ea	26,717-28,156 .	1,440	Insecticidal protein gene from *Ophioglossum pendulum* (WO Patent 2017/023486; Allen et al. 2017)
Intervening Sequence	28,157-28,173	17	DNA sequence used for cloning (synthetic)
sb-SCI-1B Terminator	28,174-29,126	953	Terminator region of the *Sorghum bicolor* (sorghum) subtilisin-chymotrypsin inhibitor 18 gene (WO Patent 2018/102131; Abbitt et al. 2018)
Intervening Sequence	29,127-29,172	46	DNA sequence used for cloning (synthetic)
Z27G Terminator	29,173-29,632	460	Terminator region from the *Zea mays* W64 line 27-kDa gamma zein gene (Das et al. 1991; Liu et al. 2016)
Intervening Sequence	29,633-29,638	6	DNA sequence used for cloning (synthetic)
UBQ14 Terminator	29,639-30,540	902	Terminator region from the *Arabidopsis thaliana* ubiquitin 14 gene (Callis et al.1995)
Intervening Sequence	30,541-30,546	6	DNA sequence used for cloning (synthetic)
In2-1 Terminator	30,547-31,040	494	Terminator region from the *Zea* mays In2-1 gene (Hershey and Stoner 1991)
Intervening Sequence	31,041-31,097	57	DNA sequence used for cloning (synthetic)
*att*B2	31,098-31,121	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway cloning system (Hartley et al. 2000; Katzen 2007)
Intervening Sequence	31,122-31,268	147	DNA sequence used for cloning (synthetic)
*att*B3	31,269-31,289 (complementary)	21	Bacteriophage lambda integrase recombination site (Cheo et al. 2004)
Intervening Sequence	31,290-31,524	235	DNA sequence used for cloning (synthetic)
FRT6	31,525-31,572	48	Modified flippase recombination target site from *Saccharomyces cerevisiae* (Proteau et al. 1986)
Intervening Sequence	31,573-31,987	415	DNA sequence used for cloning (synthetic)
Ti Plasmid Region	31,988-32,044	57	Sequence from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)
Left Border (LB)	32,045-32,069	25	T-DNA Left Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)