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Supporting document 1

Safety assessment - Application A1302

Food derived from insect-protected corn line MZIR260

Executive summary

Application A1302 seeks approval for the sale and use of food derived from corn line MZIR260 that has been genetically modified (GM) for protection against lepidopteran insect pest.

Protection against lepidopteran insect pests is conferred by the expression of the e*Cry1Gb.1Ig-03* gene encoding the insecticidal eCry1Gb.1Ig protein. The *eCry1Gb.1Ig-03* gene is composed of two sequences from *Bacillus thuringiensis*: (1) the *cry1Gb* gene; and (2) the *cry1Ig* gene. MZIR260 also expresses the phosphomannose isomerase (PMI) protein from *Escherichia coli* strain K-12 as a selectable marker. The PMI protein has been assessed previously by Food Standards Australia New Zealand (FSANZ). This is the first time FSANZ has assessed the eCry1Gb.1Ig 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 eCry1Gb.1Ig (*eCry1Gb.1Ig-03*) and PMI (*pmi-15*) were introduced into corn line MZIR260 via *Agrobacterium*-mediated transformation. Molecular analyses indicate that a single copy of each of the linked eCry1Gb.1Ig-03 and pmi-15 expression cassettes is present at a single insertion site in the MZIR260 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

Both eCry1Gb.1Ig and PMI are expressed throughout MZIR260. Expression levels are low in grain. Bioinformatic studies confirmed the lack of any significant amino acid sequence similarity between eCry1Gb.1Ig and known protein toxins or allergens. Laboratory studies demonstrated that the eCry1Gb.1Ig protein is susceptible to the action of digestive enzymes and would be thoroughly degraded before being absorbed during passage through the gastrointestinal tract. eCry1Gb.1Ig is also susceptible to heat inactivation at temperatures typically used in food processing.

Characterisation studies confirmed that the PMI protein is identical to proteins previously assessed by FSANZ. Updated bioinformatic analyses for the PMI protein are consistent with previous analyses showing that this protein has no significant homology with any known allergens or toxins.

Taken together, the evidence supports the conclusion that eCry1Gb.1Ig and PMI are not toxic or allergenic to humans.

Compositional analyses

Detailed compositional analyses were performed on MZIR260. Statistically significant differences were found between grain from MZIR260 and the control for 11 of the 58 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 MZIR260 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 insect-protected corn line MZIR260. On the basis of the data provided in the present application and other available information, food derived from MZIR260 is considered to be as safe for human consumption as food derived from conventional non-GM corn cultivars.

Table of contents

EXECUTIVE SUMMARY I

1 INTRODUCTION 4

2 HISTORY OF USE 4

2.1 HOST ORGANISM 4
2.2 DONOR ORGANISMS 5

3 MOLECULAR CHARACTERISATION 6

3.1 TRANSFORMATION METHOD 6
3.2 DETAILED DESCRIPTION OF INSERTED DNA 6
3.3 DEVELOPMENT OF THE CORN LINE FROM THE ORIGINAL TRANSFORMANT 7
3.4 CHARACTERISATION OF THE INSERTED DNA AND SITE(S) OF INSERTION 8
3.5 CONCLUSION 12

4 CHARACTERISATION AND SAFETY ASSESSMENT OF NOVEL SUBSTANCES 13

4.1 ECRY1GB.1IG 13
4.2 PMI 17
4.3 PROTEIN EXPRESSION LEVELS IN MZIR260 18

5 COMPOSITIONAL ANALYSIS 19

5.1 KEY COMPONENTS 19
5.2 STUDY DESIGN 19
5.3 ANALYSES OF KEY COMPONENTS IN GRAIN 21

6 NUTRITIONAL IMPACT 23

7 REFERENCES 24

APPENDIX 1 27

APPENDIX 2 28

Index of Figures

	Title	Page
Figure 1	Map of plasmid pSYN24795	7
Figure 2	Stages of corn growth	17
Figure 3	Analytes measured in MZIR260 grain samples	20
Figure 4	Visual summary of statistically significant compositional differences between MZIR260 and the conventional control	22 - 23

Index of Tables

	Title	Page
Table 1	Expression cassettes contained in the T-DNA of pSYN24795	7
Table 2	MZIR260 generations used for various analyses	8
Table 3	Segregation results in three generations of MZIR260	10
Table 4	Bioactivity of MZIR260- and *E. coli*-derived eCry1Gb.1Ig in a diet fed to insect larvae	14
Table 5	Bioactivity of heat-treated eCry1Gb.1Ig in a diet fed to insect larvae	16

List of Abbreviations

Abbreviation	Description
ADF	acid detergent fibre
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
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
HFCS	high fructose corn syrup
kDa	kilodalton
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOQ	limit of quantitation
mg	milligram
Min	minutes
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
ORF	open reading frame
PCR	polymerase chain reaction
PMI	phosphomannose isomerase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SMRT	single molecule, real-time
TDF	total dietary fibre
µg	microgram
USDA	United States Department of Agriculture
UTR	Untranslated region

1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from Syngenta 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 MZIR260, with the OECD Unique Identifier SYN-ØØ26Ø-3. This corn line has been modified for protection against lepidopteran insect pests, including fall armyworm.

Protection against lepidopteran insect pests is conferred by the expression of the *eCry1Gb.1Ig-03* gene encoding the insecticidal eCry1Gb.1Ig protein. The *eCry1Gb.1Ig-03* gene is composed of two DNA sequences derived from the following *Bacillus thuringiensis* genes: (1) the *Cry1Gb* gene; and (2) the *Cry1Ig* gene (Chae et al. 2022). FSANZ has assessed numerous previous applications for crops containing Cry proteins derived from *B. thuringiensis*. This is the first time FSANZ has assessed the eCry1Gb.1Ig protein.

MZIR260 also expresses the phosphomannose isomerase (PMI) protein from *Escherichia coli* strain K-12 as a selectable marker. The PMI protein has been assessed previously by FSANZ.

If approved, food derived from MZIR260 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 AX5707 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 an estimated global production of 1,230 MT[1] in 2023/24, ahead of wheat (789 MT) and rice (528 MT) (USDA 2024). 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 390 MT in 2023/24 (USDA 2024). Canada produced 15.1 MT in 2023/24 (USDA 2024). Of the corn grown in the United States and Canada, an estimated 94% and ~ 90%, respectively, is GM.[3,4] 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 2022 these amounted to 0.430 and 0.188 MT respectively (FAOSTAT 2024). To supplement their limited local production of corn, Australia and New Zealand import both corn grain and processed corn products. For example, in 2022 the imported quantities of corn flour into

[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] Statistics Canada, 2024: https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210004201

Australia and New Zealand were 14,278 and 1,723 tonnes respectively, while imports of corn oil totalled 688 and 54 tonnes respectively (FAOSTAT 2024).

Corn has a long history of safe consumption by humans.[5] 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 gene encoding the eCry1Gb.1Ig protein is derived from *Bacillus thuringiensis*, a facultative anaerobic, gram-positive spore-forming bacterium found in soil (Palma et al. 2014). *B. thuringiensis* expresses a number of insecticidal proteins, including the well-characterised Cry proteins. These toxins are highly specific to their target insects, are innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo et al. 2007).

B. thuringiensis strains have a long history of use for the control of agricultural insect pests[6] (Nester et al. 2002; CERA 2011). Currently, there are approximately 44 biopesticide products based on *B. thuringiensis* registered in Australia (APVMA 2024) and 10 in New Zealand (ACVM 2024). The widespread use of *B. thuringiensis* in agriculture, along with its ubiquitous presence in soil and on vegetation, mean humans have a long history of safe exposure through food.

B. thuringiensis has been linked to human diarrheal illness due to 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, it is unlikely *B. thuringiensis* subspecies or strains are causal agents of food-induced diarrhoea (Raymond and Federici 2017; Biggel et al. 2022).

2.2.3 Escherichia coli

The *pmi-15* 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.

2.2.2 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of MZIR260 (refer to Table 1). These genetic elements are non-coding sequences and are used to regulate the expression of *eCry1Gb.1Ig-03* and *pmi-15*.

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

Details of the 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

To create the MZIR260 corn line, the conventional AX5707 corn line was transformed using the plasmid pSYN24795 (Figure 1). The methodology is outlined in the flowchart in Appendix 1 and summarised below.

Transformation of the AX5707 line was achieved by co-culturing immature embryos excised from a post-pollinated corn ear with *Agrobacterium tumefaciens* containing the pSYN24795 plasmid (Zhong et al. 2018). Immature embryos were then placed on selective media containing timentin to suppress the growth of excess *Agrobacterium.* In addition, the PMI selectable marker was used to identify transformed cells by growing transformed plant cells on media containing mannose.

After the transformed embryos reached the callus stage, the calli were placed on media to encourage shoot and root development. Rooted plants were screened using polymerase chain reaction (PCR) to identify T0 plants carrying the transfer DNA (T-DNA) but not the plasmid backbone (Figure 1). T0 plants containing single copies of *eCry1Gb.1Ig-03* and *pmi-15* genes were transferred to the greenhouse for further propagation.

Subsequent generations were further screened using standard molecular biology techniques, allowing selection of plants with *eCry1Gb.1Ig-03* and *pmi-15* expression cassettes, but without unintended DNA insertions. Following the evaluation of insert integrity, gene expression, phenotypic characteristics and agronomic performance, corn line MZIR260 was selected.

3.2 Detailed description of inserted DNA

Corn line MZIR260 contains T-DNA from plasmid pSYN24795 (Figure 1) and includes the *eCry1Gb.1Ig-03* and *pmi-15* expression cassettes.

Information on the genetic elements in the T-DNA used for transformation is summarised in Table 1. Additional detail, including intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in Appendix 2.

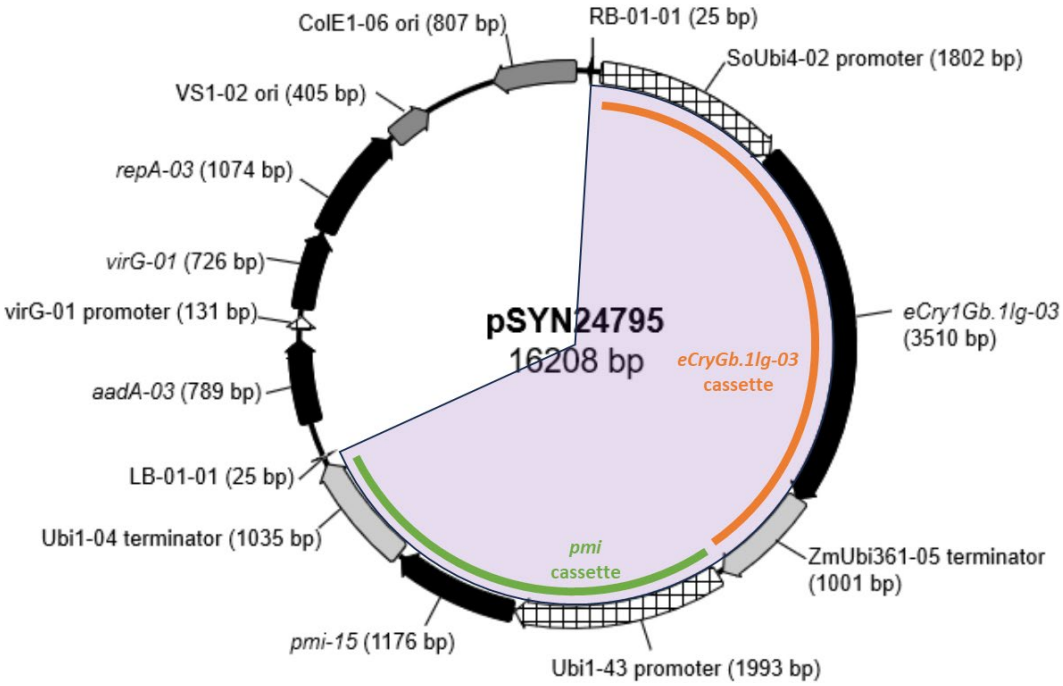


Figure 1. *Map of plasmid pSYN24795. The T-DNA region between the left and right border regions is shaded purple and was inserted into the corn genome. This region contains the eCry1Gb.1Ig-03 and pmi-15 expression cassettes. The plasmid backbone is unshaded.*

Table 1: Expression cassettes contained in the T-DNA of pSYN24795

	Promoter	Intron-containing 5'UTR	Coding sequence	Terminator
***eCry1Gb.1Ig-03* expression cassette**	Promoter region from *Saccharum officinarum* (sugar cane) *ubiquitin 4 gene*	Intron region *S. officinarum ubiquitin 4 gene*	Chimeric gene comprised of *Cry1Gb* and *Cry1Ig* gene from *B. thuringiensis*	Terminator region from *Z. mays ubiquitin gene*
***pmi-15* expression cassette**	Promoter region from *Z. mays ubiquitin 1 gene*	Intron region *Z. mays ubiquitin 1 gene*	*pmi-15* gene from *E. coli* strain K-12	Terminator region from *Z. mays ubiquitin 1 gene*

3.3 Development of the corn line from the original transformant

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of MZIR260
- ensuring that the MZIR260 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: MZIR260 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Number of integration sites	section 3.4.1	T1, T3 and F1	AX570
Absence of backbone and other sequences	section 3.4.2	T1, T3 and F1	AX5707
Insert integrity and site of integration	section 3.4.3	T3	AX5707
Genetic stability	section 3.4.4.1	T1, T3 and F1	AX5707
Mendelian inheritance	section 3.4.4.2	BC2F1, BC3F1 and F2	N/A
Expression of phenotype over several generations	section 3.4.4.2	BC2F1, BC4F1 and BC5F1	AX5707/BDAX4608
Compositional analysis	section 5	F1	AX5707/ IJ010

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

A range of analyses were undertaken to characterise the genetic modification in MZIR260. These analyses focused on the nature and stability of the insertion and whether any unintended re-arrangements or products may have occurred as a consequence of the transformation procedure.

Genomic DNA (gDNA) from MZIR260 (T1, T3 and F1 generation) and the non-GM parental line AX5707 (control) were sequenced using next generation sequencing (NGS). This method generated ~ 150 bp paired-end reads. In addition, the transformation plasmid pSYN24795 was sequenced to serve as a reference. To assess the sensitivity of the NGS method, plasmid DNA was spiked and sequenced. Sufficient sequence reads were obtained to cover the entire genomes of MZIR260 and the control, with a depth of coverage ≥168x and an adequate level of sensitivity.[7]

3.4.1 Number of integration site(s)

NGS reads from three generations of MZIR260 were mapped to the intended T-DNA insert region. For each generation, two unique insert-flank junction sites were identified. Each junction sequence comprised of the inserted T-DNA sequence joined to a flanking sequence in the corn genome. This indicates that a single copy of the T-DNA insert has been integrated into the genome of MZIR260. As expected, no junction sequences were detected in the AX5707 control.

3.4.2 Absence of backbone and other sequences

NGS reads from three generation of MZIR260 (T1, T3 and F1) and the pSYN24795 transformation plasmid were aligned. A few reads aligned to small, scattered regions of the pSYN24795 plasmid backbone that shares homology with bacterial sequences, and similar low coverage of read alignments was observed between the same region of pSYN24795 plasmid backbone and the control, thus indicating these reads were derived from other

[7] The NGS method was sufficiently sensitive to detect 100% of the spiked plasmid when present at ¼th of a copy per genome equivalent or greater. Additionally, reads were mapped to 10 single copy BUSCO (Benchmarking Universal Single-Copy Orthologues) genes and the depth of coverage for MZIR260, and the controls was comprehensive.

exogenous sources. Bacterial contaminants are common in greenhouse-procured tissue samples therefore this result is not unexpected (Zastrow-Hayes et al. 2015). No other backbone sequences including antibiotic resistance genes were identified in MZIR260 genome from this alignment.

3.4.3 Insert integrity and site of integration

The NGS analysis indicated that MZIR260 contains a single copy of the T-DNA from pSYN24795.

PacBio Single Molecule Real-Time (SMRT) sequencing analysis was performed on gDNA from MZIR260 (T3 generation) covering the full length of the insert and at least 1000 base pair (bp) of the flanking genomic sequences of MZIR260. The SMRT sequencing technology can sequence single, long DNA molecules without the requirement for DNA amplification. Sequencing is based on real-time imaging of distinct fluorescently-labelled nucleotides that are incorporated by a polymerase during DNA synthesis. This method uses circular, single stranded DNA as template which is read several times to generate the Circular Consensus Sequencing (CCS)[8] reads which has 99.9% accuracy (Rhoads and Au 2015).

Alignment of the CCS reads with the corresponding T-DNA from pSYN24795 confirmed that the T-DNA insert in MZIR260 is 10,850 bp long and the organisation is as expected, with the exception of the border regions. The inserted T-DNA had the entire right border (RB) deleted along with 16 bp of T-DNA derived intervening sequence and an 8 bp deletion of the left border (LB). In addition, there was a single nucleotide G to T change at bp 1786 identified in the intron region of sugarcane-derived ubiquitin promoter (SoUbi4-02). Neither of these changes would have a functional impact on the expression of the inserted *eCry1Gb.1Ig-03* and *pmi-15* cassettes.

The identified genomic sequences flanking the insertion site were further subjected to homology searches against the reference genome sequence of the conventional control[9] (Altschul et al. 1990). These searches located the T-DNA insert at a specific locus in the corn genome. The insertion did not disrupt any genes or any other known annotated feature in the corn genome.

To further examine the T-DNA insertion site, PCR primers flanking the insertion site were used to amplify gDNA from MZIR260 and the conventional control. Comparing the products from MZIR260 and the control identified a 30 bp deletion of the corn genomic DNA that occurred during T-DNA integration. Such changes are common during *Agrobacterium*-mediated plant transformation due to double-strand break repair mechanisms (Gheysen et al. 1991; Mayerhofer et al. 1991; Gelvin 2021).

3.4.4 Stability of the genetic changes in corn line MZIR260

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.

[8] CCS reads are consensus sequences resulting from the alignment of multiple subreads from a single polymerase read.
[9] Maize (B73) Public Genome Assembly: https://www.ncbi.nlm.nih.gov/assembly/GCF_902167145.1/

3.4.4.1 Genetic stability

NGS was used to show the genetic stability of the inserted DNA in MZIR260 by evaluating leaf-derived gDNA from three generations of MZIR260 (T1, T3, and F1). Genomic DNA from the control served as a negative control, and the control DNA spiked with plasmid pSYN24795 (Figure 1) served as a positive control.

The analysis showed the two insert-flank junction sequences present in all three generations were identical. No other junction sequences were present. The consistency of these results confirmed that the single T-DNA insert is stably maintained in corn line MZIR260.

3.4.4.2 Phenotypic stability

Mendelian inheritance

Since the inserted DNA resides at a single locus within the MZIR260 genome, it would be expected to be inherited according to Mendelian principles. To confirm this, three generations of MZIR260 (BC2F1, BC3F1 and F2) were tested for the presence of e*Cry1Gb.1Ig-03* and *pmi-15* genes using real-time quantitative PCR (RT-qPCR) analysis. A Pearson's chi-square (χ^2) analysis was conducted to compare the observed and expected segregation ratios across these generations.

According to Mendelian inheritance principles, the expected segregation ratios for the BC2F1 and BC3F1 generations is 1:1 and for the F2 generation is 1:2:1. The χ^2 critical value to reject the hypothesis of this ratio at a 5% level of significance was 3.84 for BC2F1 and BC3F1 generation and 5.99 for the F2 generation (Strickberger 1976). The results of the χ^2 analyses on all three generations tested gave χ^2 values < 3.84 for BC2F1 and BC3F1 generations and < 5.99 for the F2 generation (Table 3), indicating there were no significant differences between the observed and expected segregation ratios in any of the generations. These data support the conclusion that the inserted DNA is present at a single locus in MZIR260 genome and is inherited predictably according to Mendelian inheritance principles.

Table 3: Segregation results in three generations of MZIR260

Generation	Expected segregation ratio	Observed number of plants				Statistical analysis	
		Homozygous Positive	Hemizygous positive	Homozygous Negative	Total	χ^2	*P* value
BC2F1	1:1	98	0	112	210	0.933	0.627
BC3F1	1:1	117	0	101	218	1.174	0.556
F2	1:2:1	26	66	40	132	2.970	0.227

Expressed phenotype over several generations

The expression of the eCry1Gb.1Ig and PMI proteins in three generations of MZIR260 (BC2F1, BC4F1 and BC5F1) was examined. Enzyme-linked immunosorbent assay (ELISA) was performed on leaves, roots, pollen, forage and grain tissues from each generation, with non-GM conventional control tissues used as negative controls.

In all three generations, the expression of eCry1Gb.1Ig and PMI proteins in MZIR260 were consistent. None of the proteins were detected in the tissues from the control. These data support the conclusion that the eCry1Gb.1Ig and PMI proteins are stably expressed over multiple generations.

3.4.5 Open reading frame analysis

A bioinformatic analysis of the MZIR260 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel open reading frames (ORFs) had been created in MZIR260 as a result of the DNA insertion, and whether any putative peptides encoded by the identified ORFs have the potential for allergenicity or toxicity.

Sequences spanning the 5' and 3' insert-flank junctions of MZIR260 were translated *in silico* from start codon (ATG) to stop codon (TGA, TAG, TAA) in all six reading frames. A total of 35 ORFs were identified from the inserted sequence that correspond to putative peptides of 30 amino acids or greater in length were investigated further to determine whether their amino acid sequence showed similarity with known allergen and toxin peptide sequences in established databases. There were no junction putative ORFs identified in MZIR260.

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 putative peptides identified above were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource (COMPARE[10]) database, from the Health and Environmental Science Institute. At the date of the search (March 2023), there were 2,631 sequences in the allergen database.

A FASTA search algorithm (v36.3.8) was used to identify alignments between query sequences and the COMPARE database, using a BLOMSUM50 scoring matrix. For translated ORFs over ≥ 80 amino acids only matches of greater than 35% similarity were considered. For translated ORFs with < 80 (but ≥ 30) amino acids, the E-value of $1x10^{-6}$ was used to identify potential relevant alignments.

An 8-mer exact match search for ≥ 8 contiguous amino acids match to the allergens from the COMPARE database was performed using an in-house algorithm. Only matches of 100% similarity over 8 amino acids were considered.

No matches between the 35 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 not considered an indication of allergenic risk as there are no known cross-reactive allergens that share an 8-amino-acid contiguous match without also sharing > 35% identity across an 80 amino acid window (Carlson et al. 2023). Additionally, the source organism for PMI, *Escherichia coli* is not known to have intrinsic allergens, and numerous previous FSANZ safety assessments[11] have demonstrated that PMI has extensive history of safe use in GM crops.

Given these results, risk of allergenic proteins with relevance to human safety being produced by the ORFs in MZIR260 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*

[10] http://comparedatabase.org/database/
[11] A564, A580, A1001, A1138, A1060, A1202, A1270, A1272, A1281.

to an in-house toxin database (March 2023). This database is a subset of sequences derived from the UniProt Knoweldgebase[12] database, curated to remove *B. thuringiensis* Cry protein entries, which have been demonstrated to be non-toxic to mammals (Betz et al. 2000) as well as any likely non-toxin proteins. The database contained 9,053 sequences at the date of analysis. A BLASTP algorithm with a BLOSUM62 scoring matrix and an E-value of ≤ $1x10^{-5}$ was used.

No alignments with an E-value of ≤ $1x10^{-5}$ were identified, indicating there was no significant homology between the putative peptides and any known protein toxins.

3.5 Conclusion

Corn line MZIR260 contains a single copy of the intended DNA insertion, integrated at a specific locus in the corn genome. Sequencing results confirmed that the *eCry1Gb.1Ig-03* and *pmi-15* expression cassettes were inserted with the expected organisation. No plasmid backbone sequences, including any antibiotic resistance genes from the plasmid used in the transformation, are present.

The inserted DNA is stably inherited and expressed across several breeding generations of MZIR260. None of the new ORFs created by the insertion raise any allergenicity or toxicity concerns.

[12] https://www.uniprot.org/

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.

Two novel substances are expressed in MZIR260: the eCry1Gb.1Ig insecticidal protein, which provides protection against lepidopteran pests; and the PMI protein which allows for growth on media containing mannose and acts as a selectable marker during the transformation process.

4.1 eCry1Gb.1Ig

Cry proteins are contact pesticides, requiring ingestion by the target pest[13] and passage into the digestive system in order to exert their effect (Jurat-Fuentes and Crickmore 2017). Alkaline conditions and proteases in the insect midgut cause the proteolytic cleavage of the Cry protein's protoxin domain and activation of the insecticidal toxin. The activated protein functions by binding to a highly specific glycoprotein receptor on the surface of midgut epithelial cells, aggregating and forming pores in the cell membrane (Schnepf et al. 1998). This leads to loss of cell integrity in the midgut, leading to developmental delays (growth inhibition) and insect death.

The high specificity of Cry proteins for particular target insects has made them a popular tool in GM crops to manage insect pests (Zwack et al. 2024). However, 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. The diversity of the Cry family of proteins arises from three structural domains that evolved independently, 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).

eCry1Gb.1Ig is a chimeric protein composed of the *Cry1Gb* and *Cry1Ig* genes (Chae et al. 2022). The eCry1Gb.1Ig protein is designed with the goal to achieve high levels of activity against lepidopteran insect species by disrupting the midgut epithelium in these species.

The *eCry1Gb.1Ig-03* gene prepared by the applicant encodes a protein of 1169 amino acids, with an apparent molecular weight of ~ 133 kilodalton (kDa).

4.1.1 Characterisation of eCry1Gb.1Ig expressed in MZIR260 and equivalence to a

[13] Lepidoptera, Coleoptera, Diptera and other invertebrates such as nematodes.

bacterially-produced form

The plant-derived eCry1Gb.1Ig was purified from MZIR260 leaf tissues using immunoaffinity chromatography. The purified fractions containing eCry1Gb.1Ig were combined and subsequently concentrated. To obtain sufficient quantities of eCry1Gb.1Ig for use in safety studies, eCry1Gb.1Ig was also expressed in *Escherichia coli*. The *E. coli*-derived eCry1Gb.1Ig was purified by pellet washing and size-exclusion chromatography.

The equivalence of the MZIR260- and *E. coli*-derived eCry1Gb.1Ig protein must be established before the safety data generated using *E. coli*-derived eCry1Gb.1Ig can be applied to MZIR260-derived eCry1Gb.1Ig. In order to confirm the identity and equivalence of the MZIR260- and *E. coli*-derived eCry1Gb.1Ig, a series of analytical techniques were done, the results of which are summarised below.

Molecular weight and Immunoreactivity. Following SDS-PAGE, protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane via electroblotting. The membranes for the proteins were then probed with a protein-specific polyclonal goat primary antibody followed by an alkaline phosphatase-conjugated donkey anti-goat secondary antibody. Proteins were visualised following addition of the chromogenic substrate BCIP®/NBT.

Western blot analysis with a eCry1Gb.1Ig-specific antibody showed that the protein being expressed in MZIR260 and *E. coli* was indeed eCry1Gb.1Ig and they have equivalent immunoreactivity. Both MZIR260- and *E. coli*-derived eCry1Gb.1Ig proteins showed immunoreactive bands corresponding to the anticipated MW of approximately 133 kDa. A faint band of ~49 kDa was also observed in both fractions and in the non-GM control spiked with *E. coli*-derived eCry1Gb.1Ig protein, but not in the non-GM control. This likely represents degraded eCry1Gb.1Ig protein fragments.

Peptide mapping. MZIR260- and *E. coli*-derived eCry1Gb.1Ig proteins were digested with trypsin or chymotrypsin and analysed via liquid chromatography-tandem mass spectrometry (LC-MS/MS). For MZIR260-derived eCry1Gb.1Ig, the combined tryptic and chymotryptic peptides covered 88% of the expected protein sequence.[14] For the *E. coli*-derived eCry1Gb.1Ig the peptide coverage was 96% of the expected protein sequence.

N- and C-terminal sequencing. The N and C-terminal peptide of MZIR260- and *E. coli*-derived eCry1Gb.1Ig were identified by peptide mass coverage analysis and were identical with each other and consistent with the expected protein sequence.

Glycosylation analysis. SDS-PAGE combined with a colourimetric glycoprotein detection procedure showed that the eCry1Gb.1Ig protein from both MZIR260 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 MZIR260- and *E. coli*-derived eCry1Gb.1Ig was evaluated in an insect bioassay using soybean looper (SBL) larvae, a lepidopteran insect which is sensitive to the eCry1Gb.1Ig protein.

In this assay, SBL larvae were fed a diet mixture containing MZIR260 crude leaf extract, *E. coli*-derived eCry1Gb.1Ig and non-GM control crude leaf extract spiked with *E. coli*-derived eCry1Gb.1Ig protein. The extracts were diluted in buffer to final concentrations ranging from

[14] The expected sequence refers to the translated sequence for the gene derived from the expression vector and used for production of the *E. coli*-derived eCry1Gb.1Ig protein.

0.0078 - 150 µg/ml eCry1Gb.1Ig protein. Negative controls comprised of non-GM control extract and plain buffer and were similarly incorporated into the SBL diet. The bioassays were conducted in 24-well culture plates with each well containing one SBL larva and 800 µL diet with 50 µL of test/control solutions. Mortality readings were taken periodically starting at 72 hours and continued until 168 hours. LC_{50}[15] determinations, where possible, were assessed.

The results of the bioassay showed that the LC_{50} value for *E. coli*-derived eCry1Gb.1Ig protein was very similar to that of the MZIR260-derived eCry1Gb.1Ig protein and the non-GM control spiked with *E. coli*-derived eCry1Gb.1Ig protein, with overlapping 95% confidence interval (CI) (Table 4). The results demonstrate that MZIR260- and *E. coli*-derived eCry1Gb.1Ig protein are functionally active against the target insect SBL.

Table 4: Bioactivity of MZIR260- and *E. coli*-derived eCry1Gb.1Ig in a diet fed to insect larvae

Treatment	LC_{50} (ng/cm^2)	95% CI (ng/cm^2)	Slope ± SEM
E. coli-derived eCry1Gb.1Ig protein	2.42	1.98 – 2.9	1.88 ± 0.152
MZIR260-derived eCry1Gb.1Ig protein	2.84	2.33 – 3.38	2.14 ± 0.191
Non-GM control crude extract spiked with *E. coli*-derived eCry1Gb.1Ig protein.	2.69	2.23 – 3.18	2.25 ± 0.196

The results outlined in this section demonstrate that *E. coli*-derived eCry1Gb.1Ig is functionally and structurally equivalent to MZIR260-derived eCry1Gb.1Ig. *E. coli*-derived eCry1Gb.1Ig is therefore a suitable surrogate for use in the safety assessment experiments described in section 4.1.2.

4.1.2 Safety of the introduced eCry1Gb.1Ig

Data were provided to assess the potential allergenicity and toxicity of eCry1Gb.1Ig.

Bioinformatic analyses of eCry1Gb.1Ig

Bioinformatic analyses, as described in section 3.4.5.1, compared the eCry1Gb.1Ig amino acid sequence to known allergenic proteins in the COMPARE Allergen database (2023). The search identified 43 alignments with E-values ≤ 10. None of the 43 alignments met or exceeded the threshold of greater than 35% similarity over ≥ 80 amino acids, and no eight amino acid peptide matches were shared between the eCry1Gb.1Ig sequence and proteins in the allergen database.

To assess the similarity of eCry1Gb.1Ig to known toxins, the applicant provided results of i*n silico* analyses comparing the eCry1Gb.1Ig amino acid sequence to proteins in the National Center for Biotechnology Information (NCBI)[16] database (August 2023) and to proteins identified as “toxins” in the in-house Toxin database described in section 3.4.5.2. A BLASTP algorithm with a BLOSUM62 scoring matrix and an E-value of ≤ $1x10^{-5}$ was used. The search did not identify any known toxins with homology to eCry1Gb.1Ig.

[15] LC_{50} = 50% lethal concentration
[16] https://www.ncbi.nlm.nih.gov/

Susceptibility of eCry1Gb.1Ig to digestion with pepsin

E. coli-produced eCry1Gb.1Ig (test protein) was incubated at 37°C ± 2°C in a simulated gastric fluid (SGF) system containing pepsin (10U enzyme/ug protein) at an acidic pH of ~1.2 for 0 - 60 min (Thomas et al. 2004). Controls included a no test protein control (pepsin only) and no pepsin control (test protein only) incubated for 0 and 60 min. Bovine serum albumin (BSA) and β-lactoglobulin were used as positive and negative controls respectively. The extent of digestion was visualised by SDS-PAGE with Coomassie Blue staining followed by Western blotting.

The results from the pepsin digestion showed that by one min, there was no intact eCry1Gb.1Ig remaining in the reaction mix. Some low molecular weight (~3 - 4 kDa) bands were detectable by SDS-PAGE (but not by Western blot) after one minute of eCry1Gb.1Ig digestion and were present throughout the 60 min time course. The BSA control was rapidly digested by 1 min and the β-lactoglobulin control remained present over the course of the reaction. eCry1Gb.1Ig remained intact after 60 min in buffer and in the gastric solution without pepsin. These results indicate that eCry1Gb.1Ig is rapidly digested by pepsin.

Bioactivity of eCry1Gb.1Ig after exposure to heat

The thermal stability of eCry1Gb.1Ig was evaluated by assessing the functional activity of the heat-treated eCry1Gb.1Ig protein in an insect bioassay. *E. coli*-derived eCry1Gb.1Ig protein was incubated for 30 min at 25°C, 37°C, 65°C, or 95°C before incorporation into an artificial diet for *Spodoptera frugiperda* larvae. The control diet contained either unheated eCry1Gb.1Ig (kept at 4°C) or the buffer used to dilute the eCry1Gb.1Ig protein (negative control). The test diets contained a final concentration of 497 ng eCry1Gb.1Ig / cm^2 diet surface. Each diet was provided to 144 individual *S. frugiperda* larvae (except for the 65°C test sample and the negative control diet which was provided to 143 and 72 larvae respectively) for a total of 5 days. Mortality was assessed after day 5.

Table 5: Bioactivity of heat-treated eCry1Gb.1Ig in a diet fed to insect larvae

Treatment description	Incubation condition	Number of observations	Total number of surviving organisms	Mortality (%)	p-value[2]
Negative control	-	72	0	0	-
Unheated control	4°C	144	21	85.4	-
Test diet	25°C	144	26	81.9	0.0829
	37°C	144	107	50.7	**<0.0001**
	65°C	143	100	30.1	**<0.0001**
	95°C	144	142	1.4	**<0.0001**

The results demonstrated that when heated to temperatures of 37°C - 95°C for ~30 minutes, the bioactivity of eCry1Gb.1Ig was effectively reduced, with 1.4% mortality observed for the larvae fed diets containing eCry1Gb.1Ig heat-treated at 95°C (Table 5). Dunnett's t-test was used to determine whether the mortality rate of *S. frugiperda* that had been fed diets containing heated eCry1Gb.1Ig was smaller than that of those fed the unheated control diet. For eCry1Gb.1Ig heated to 37°C - 95°C, the decrease in activity against *S. frugiperda* larvae was statistically significant (p-value <0.05) compared to the unheated control. eCry1Gb.1Ig 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 eCry1Gb.1Ig is heat labile at temperatures ≥ 37°C.

4.1.3 Conclusion

A range of characterisation studies were performed on MZIR260-derived eCry1Gb.1Ig confirming its identity, structure and function as well as equivalence to a bacterially expressed eCry1Gb.1Ig. Bioinformatic analyses showed eCry1Gb.1Ig does not share any meaningful homology with any known allergens or toxins. eCry1Gb.1Ig is heat labile at ≥ 37°C and susceptible to digestion by pepsin. Taken together this indicates that the eCry1Gb.1Ig protein is unlikely to be toxic or allergenic to humans.

4.2 PMI

The *pmi-15* gene in MZIR260 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-15* gene encodes a 391 amino acid protein with a calculated molecular weight of ~43 kDa. PMI has been assessed by FSANZ previously as a novel protein in 7 corn lines and one rice line.

4.2.1 Safety of the introduced PMI

The PMI protein has been previously assessed by FSANZ in 8 corn lines,[17] as well as in rice line GR2E.[18] These previous assessments did not raise any safety concerns and there are

[17] 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), DP915635 (Application A1272; FSANZ 2024) and DP910521 (A1281; FSANZ 2024).
[18] Rice line GR2E - Application A1138; FSANZ 2017.

no credible reports of adverse health effects in humans. Since the PMI protein expressed in MZIR260 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 (August 2023) for PMI that looked for amino acid sequence similarity to known protein allergens and toxins were provided by the applicant. The results do not alter conclusions reached in previous assessments.

4.2.2 Conclusion

The data presented by the applicant confirms the PMI expressed in MZIR260 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.

4.3 Protein expression levels in MZIR260

For analysis of the expression levels of the eCry1Gb.1Ig and PMI proteins in MZIR260, tissues were collected from four field-trial sites in representative corn-producing regions of the United States during the 2022 growing season.[19] Tissues were collected at varying stages of growth (see Figure 2 for a summary of corn growth stages). Tissues were lyophilised, homogenised (except pollen samples) and stored frozen until analysis.

eCry1Gb.1Ig and PMI were extracted from tissues using standard methods and their expression levels quantified in each tissue using a quantitative ELISA. For each tissue analysed, five samples were processed from each of the four field-trial sites (except for pollen tissue – one pooled sample from 20 tassels from each field-trial site).

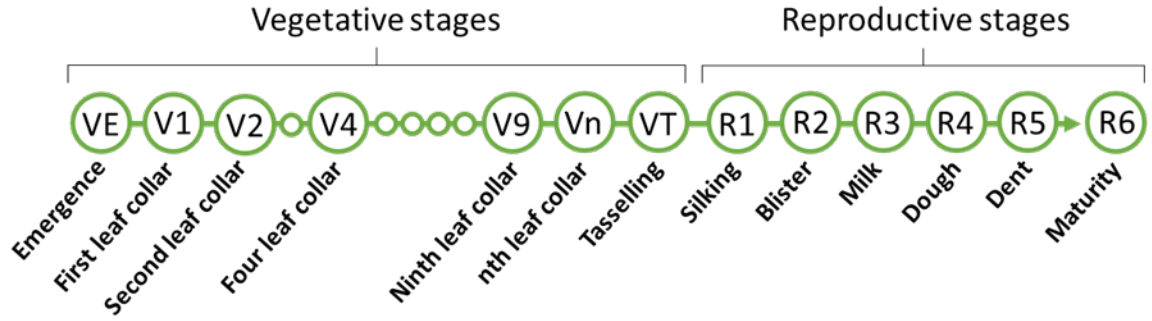


Figure 2. *Stages of corn growth. Grain is harvested at maturity (R6).*

Results from the ELISA showed that for eCry1Gb.1Ig, the highest expression levels in MZIR260 were in pollen (773 µg/g DW). The lowest expression was in whole plant (198 ug/g DW). PMI had the lowest expression levels in all tissues and was expressed at particularly low levels in leaf (4.78 µg/g DW), root (5.95 µg/g DW) and grain (6.73 µg/g DW).

For the full set of expression data, including standard deviations and ranges, refer to the Application dossier[20] (pages 66 – 68).

[19] The location of the four field trial sites: one site in Pennsylvania, Nebraska, Illinois and Iowa.

[20] https://www.foodstandards.gov.au/food-standards-code/applications/a1302-food-derived-insect-protected-corn-line-mzir260

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 transgenic 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.

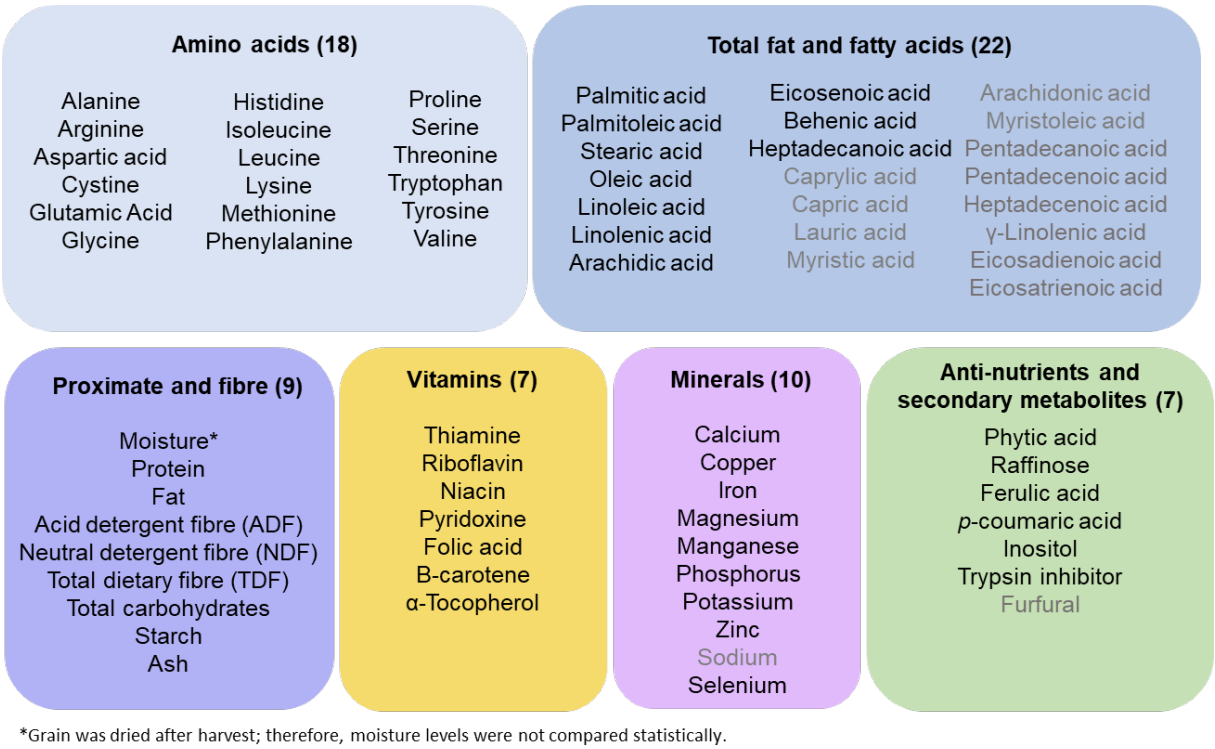
5.2 Study design

MZIR260 (F1 generation), a non-GM near isogenic control corn line, and a total of 6 non-GM commercial reference hybrid lines were grown and harvested from eight field trial sites in the United States during the 2022 growing season.[21] 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 MZIR260, control, and three reference lines selected from NK1066, NK0281, NK9813, NK1284, NK0760 and NK1452. Plants were grown under agronomic field conditions typical for each growing region.

At maturity (R6 growth stage), grain was harvested from all plots, dried and shipped to analytical laboratory at ambient temperature, before being frozen at -20°C ± 10°C until analysis. 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.

A total of 73 analytes in grain were assessed (see Figure 3 for a complete list). For 15 of these analytes (listed in grey in Figure 4) all samples of both MZIR260 and control were below the assay limit of quantification (LOQ) and were therefore not analysed statistically.

[21] The location of the eight field trial sites: one site in Indiana, Pennsylvania, Nebraska, Wisconsin, Illinois and Minnesota; two sites in Iowa.



*Grain was dried after harvest; therefore, moisture levels were not compared statistically.

Figure 3. *Analytes measured in MZIR260 grain samples.*

The data for the remaining 58 analytes were therefore analysed. Statistical analyses were performed using Statistical Analysis Software (SAS) 9.4 (SAS Institute, Cary, North Carolina 2012). For each analyte, 'descriptive statistics' (mean and standard error) were generated. A mixed model Analysis of Variance (ANOVA) was used for both across-location and within-location comparisons. For each analyte, t-tests were used to assess the statistical significance of the MZIR260 and the control. In assessing the significance of any difference between the mean analyte value for MZIR260 and the control, a P-value of 0.05 was used.

For analytes having some of the individual sample values below the LOQ (calcium, α-tocopherol and β-carotene), the distributions among the locations and entries were scrutinised to determine if substitution of calculated values equal to the LOQ value for the sample adjusted for sample moisture was possible without exerting undue influence on the statistical analyses. This value is referred to as the "value equivalent to the LOQ," as it is not the actual method LOQ, but a calculated value.

Selenium had 66 samples <LOQ and raffinose had 6 samples <LOQ where most of the <LOQ observations occurred at particular field trial locations (selenium: 4 sites; raffinose: 1 site). Substitution of this many <LOQ observations from anyone location would affect the validity of the statistical analyses. Therefore, for selenium and raffinose, the affected field trial sites were excluded from both the across-location and within location statistical analyses.

Any statistically significant differences between MZIR260 and the control were compared to the ranges of values from the non-GM reference hybrid lines grown concurrently in the same trial as MZIR260. This reference range is useful to define the variability in corn varieties grown under the same agronomical conditions. In addition, the natural variation from the Agriculture & Food Systems Institute (AFSI) Crop Composition Database was also considered (AFSI 2024). These data ranges assist with determining whether any statistically significant differences are likely to be biologically meaningful.

Key analyte levels (proximates and minerals) were also analysed in forage but the results are

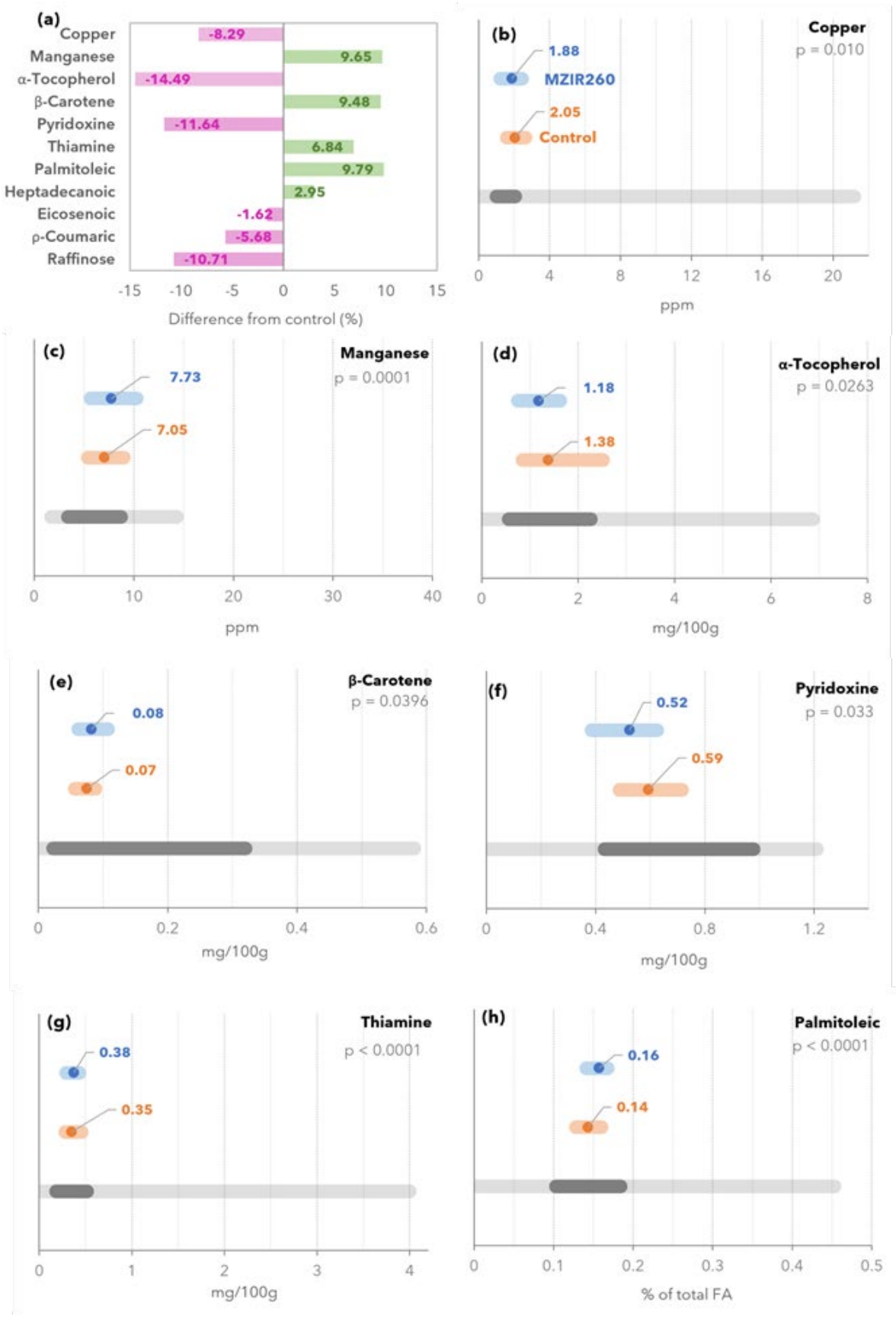
not included in this report.

5.3 Analyses of key components in grain

Of the 73 analytes measured in grain, mean values were provided for 58 analytes and of these, there were 11 for which there was a statistically significant difference ($p < 0.05$) between corn line MZIR260 and the control: copper, manganese, β-carotene. α-tocopherol, pyridoxine, thiamine, palmitoleic acid, heptadecanoic acid, eicosenoic acid, *ρ*-coumaric acid and raffinose. A summary of these 11 analytes is provided in Figure 4. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the Application dossier (pages 108 – 120).

For all 11 analytes where a statistically significant difference was found, the deviation of the MZIR260 mean from the control mean was less than 15% (Figure 4a). As can be observed in Figure 4 (panels b-l), the MZIR260 mean for each of these 11 components was within the control range value, indicating that MZIR260 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. In addition, the observed MZIR260 means fall well within the natural variability seen in the in-study reference range and/or in the publicly-available data ranges (dark grey and light grey bars respectively, Figure 4, b-l). 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 MZIR260 when compared to conventional non-GM corn cultivars already available in agricultural markets. Grain from MZIR260 can therefore be regarded as equivalent in composition to grain from conventional non-GM corn.


(a)
Copper
Manganese
α-Tocopherol
β-Carotene
Pyridoxine
Thiamine
Palmitoleic
Heptadecanoic
Eicosenoic
p-Coumaric
Raffinose
-8.29
9.65
-14.49
9.48
-11.64
6.84
9.79
2.95
-1.62
-5.68
-10.71
-15 -10 -5 0 5 10 15
Difference from control (%)
(b)
Copper
p = 0.010
1.88
MZIR260
2.05
Control
0 4 8 12 16 20
ppm
(c)
Manganese
p = 0.0001
7.73
7.05
0 10 20 30 40
ppm
(d)
α-Tocopherol
p = 0.0263
1.18
1.38
0 2 4 6 8
mg/100g
(e)
β-Carotene
p = 0.0396
0.08
0.07
0 0.2 0.4 0.6
mg/100g
(f)
Pyridoxine
p = 0.033
0.52
0.59
0 0.4 0.8 1.2
mg/100g
(g)
Thiamine
p < 0.0001
0.38
0.35
0 1 2 3 4
mg/100g
(h)
Palmitoleic
p < 0.0001
0.16
0.14
0 0.1 0.2 0.3 0.4 0.5
% of total FA

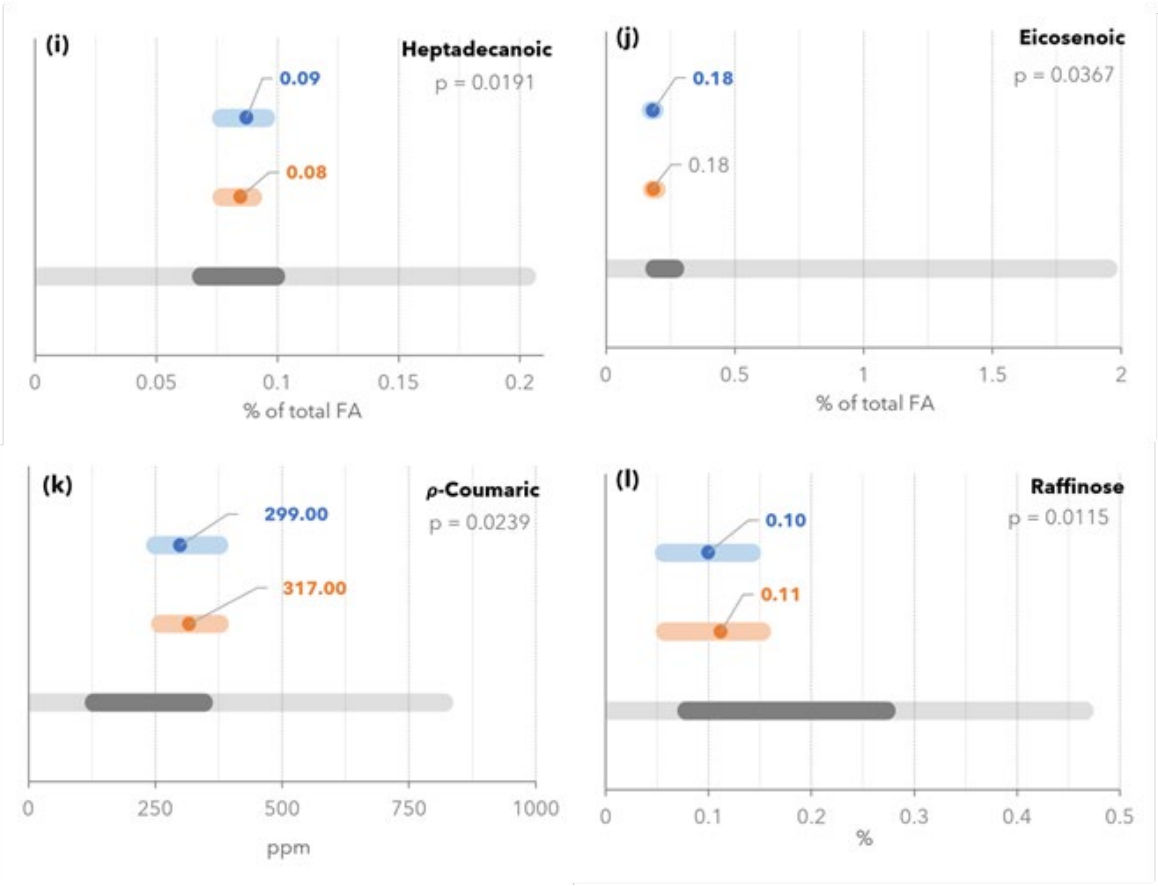


Figure 4. *Visual summary of statistically significant compositional differences between MZIR260 and the conventional control.* **(a)** *Percentage deviation of the mean MZIR260 value from the mean control value for each of the 11 analytes for which a statistically significant difference was found.* **(b) – (l)** *Measured means (dots) and ranges (coloured bars) for MZIR260 (blue) and the conventional control (orange) for the 11 analytes as labelled. The dark and grey bars represent the in-study reference range of values and publicly-available range of values, respectively, 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.

MZIR260 is the result of a genetic modification for protection against 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 of MZIR260 compared with conventional non-GM corn cultivars. The introduction of food derived from MZIR260 into the food supply is therefore expected to have negligible nutritional impact.

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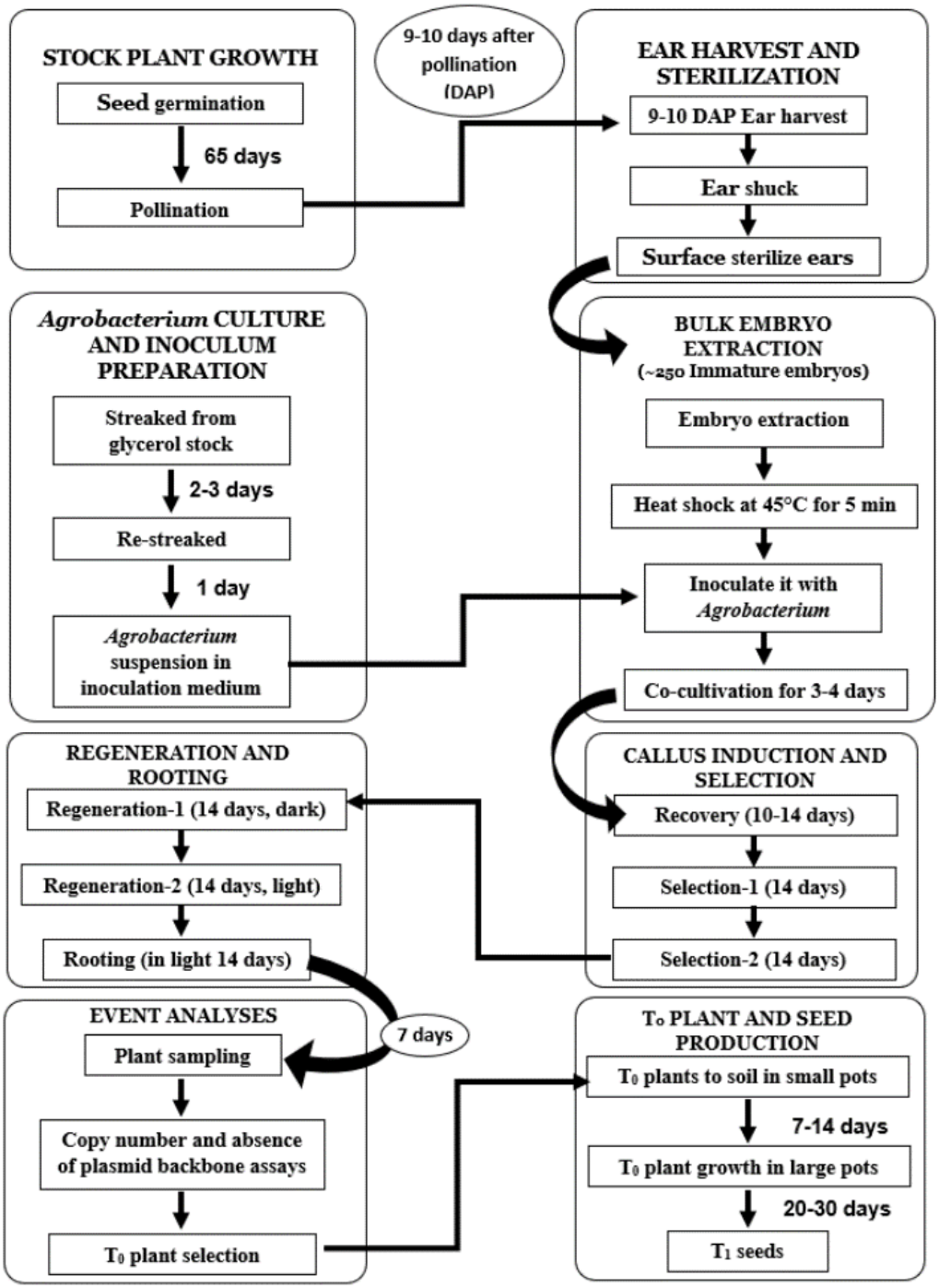
Appendix 1

Flowchart showing the development process used for creation of MZIR260 corn



Appendix 2

pSYN24795-derived genetic elements in T-DNA region

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 (GenBank ID: J01826.1) (Wang et al. 1984)
Intervening Sequence	26-116	91	DNA sequence used for cloning
***eCry1Gb.1Ig-03* expression cassette**			
SoUbi4-02 Promoter	117-1918	1802	Promoter region from the *Saccharum officinarum* (sugar cane) ubiquitin 4 containing the first intron (GenBank ID: AF093504.1 (Wei et al. 1999)
Intervening Sequence	1919-1930	12	DNA sequence used for cloning
eCry1Gb.1Ig-03	1931-5440	3510	Chimeric gene comprised of sequences from the *Cry1Gb* gene and *Cry1Ig* gene, both from *Bacillus thuringiensis* (Chae et al. 2022)
Intervening Sequence	5441-5446	6	DNA sequence used for cloning
ZmUbi361-05 Terminator	5447-6447	1001	Terminator region from the *Z. mays* ubiquitin gene (Genbank ID: U29162.1) (Nuccio 2018)
Intervening Sequence	6448-6503	56	DNA sequence used for cloning
***pmi-15* expression cassette**			
ZmUbi1-43 Promoter	6504-8496	1993	Promoter region from the *Z. mays* ubiquitin 1 gene containing the first intron (GenBank ID: S94464.1) (Christensen et al. 1992)
Intervening Sequence	8497-8508	12	DNA sequence used for cloning
pmi-15	8509-9684	1176	Escherichia coli gene *pmi* encoding the enzyme PMI (GenBank ID: M15380.1) – catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate (Negrotto et al. 2000).
Intervening Sequence	9685-9722	38	DNA sequence used for cloning
Zmubi1-04 Terminator	9723-10757	1035	Terminator region from the *Z. mays* ubiquitin 1 gene (GenBank ID: S94464.1) (Christensen et al. 1992)
Intervening Sequence	10758-10874	117	DNA sequence used for cloning (synthetic)
Left Border (LB)	10875-10899	25	T-DNA Left Border from the *A. tumefaciens* Ti plasmid (GenBank ID: J01825.1) (Yadav et al. 1982).