Lepidopteran-Protected Maize

Application to Food Standards Australia New Zealand for the Inclusion of MZIR260 Maize in Standard 1.5.2 – Food Derived from Gene Technology

Submitted by

Syngenta Australia Pty Ltd

Submitted to:

Standards Management Officer Food Standards Australia New Zealand

May 2024

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EXECUTIVE SUMMARY

Syngenta Crop Protection, LLC. has developed a genetically modified maize (*Zea mays* L.) using the techniques of modern molecular biology, to produce Event MZIR260 (OECD ID - SYN-ØØ26Ø-3) to provide control of fall armyworm (FAW, *Spodoptera frugiperda*).

Insect pests cause immense agronomic losses worldwide. One of the most destructive pests of maize crops is FAW, *Spodoptera frugiperda*. The ability to migrate long distances, a prodigious appetite, and a demonstrated ability to develop resistance to insecticides, makes it a difficult target to control. Insecticidal proteins, for example those produced by the bacterium *Bacillus thuringiensis* (*B. thuringiensis* or *Bt*), are among the safest and most effective insect control agents. GM crops expressing such proteins are a key part of a successful integrated pest management (IPM) program for FAW, *Spodoptera frugiperda*. However, the rise of FAW, *Spodoptera frugiperda*. populations that are resistant to these GM interventions highlights an urgent need for the development and commercialisation of new GM traits to effectively manage this pest (Fabrick and Wu, 2023; Zwack *et al*., 2024).

Maize plants derived from transformation Event MZIR260 produce the insecticidal protein eCry1Gb.1Ig encoded by the gene *eCry1Gb.1Ig-03*. The protein eCry1Gb.1Ig was engineered to have improved insecticidal activity against FAW, *Spodoptera frugiperda*. MZIR260 maize plants also produce the enzyme phosphomannose isomerase (PMI), encoded by the gene *pmi-15*. PMI enables transformed plant cells to utilize mannose as a primary carbon source; it was used as a selectable marker in the development of MZIR260 maize.

Transformation of *Zea mays* to produce Event MZIR260 maize was accomplished using *Agrobacterium tumefaciens*-mediated transformation of immature embryos, as described by Zhong *et al*., 2018). The transformation plasmid pSYN24795 used to generate Event MZIR260 consists of two expression cassettes and backbone sequences.

Genetic analysis confirmed the integrity of a single MZIR260 insert. The integration of the MZIR260 insert did not disrupt any known maize endogenous genes or generate any new ORFs with risk of allergenicity or toxicity to human or animal consumers. No unintended changes leading to safety concerns were identified during the molecular characterization of the MZIR260 maize. The transgenes *eCry1Gb.1Ig-03* and *pmi-15* were shown to be stably inherited and expressed over multiple generations.

The Newly Expressed Proteins (NEPs) in Event MZIR260 maize were assessed for toxicity and allergenicity risks using a weight-of-evidence approach. This approach incorporates information on the source organism, history of safe use (HOSU), *in silico* comparison to known and putative allergens and toxins, and/or stability under heat and digestive condition.

Neither eCry1Gb.1Ig or PMI proteins were found to share biologically relevant amino acid similarity to known or putative protein toxins or allergens of mammalian significance. Both proteins readily degrade and are inactivated under simulated mammalian gastric condition, and when exposed to heat. Cry and PMI proteins have a demonstrated HOSU in commercial GM crops (ISSAA, 2024). In combination with well characterised modes of action, both eCry1Gb.1Ig and PMI were considered unlikely to be food toxins or allergens. Additionally, their low expression levels in maize tissues result in minimal anticipated exposure. All these factors support the prediction that no adverse health effects will result from the exposure to the eCry1Gb.1Ig and PMI proteins expressed in MZIR260 maize. Therefore, a reasonable

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certainty exists that exposure to MZIR260 maize and the NEPs eCry1Gb.1Ig and PMI, will not result in harm to consumers.

Furthermore, the compositional analysis established nutritional adequacy. No biologically relevant impact was observed on the nutritional status of forage and grain derived from MZIR260 maize as a result of the transformation process.

Overall, the data and information presented in this application support the conclusion that MZIR260 maize is comparable to, and as safe as, conventional maize, and that no adverse health effects will result from the consumption of MZIR260 maize or maize products.

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TABLE OF CONTENTS

CONFIDENTIALITY STATEMENT **2**
EXECUTIVE SUMMARY **3**
LIST OF TABLES **9**
LIST OF FIGURES **11**
PART 1 GENERAL REQUIREMENTS **13**
1.1 Applicants and Developers Details 13
1.2 Purpose of the Application 13
1.3 Justification of the Application 13
a) Need for the Proposed Change 13
b) Advantage of the GMO 13
1.4 Regulatory Impact Information 14
1.5 Costs, Benefits, and Impact on Trade 14
1.6 Exclusive Capturable Commercial Benefits (ECCB) 14
1.7 Confidential Commercial Information 14
1.8 International and Other Standards 14
1.9 Statutory Declaration 16
1.10 Checklist 17
1.11 References Cited in Part 1 19
Article II. PART A. TECHNICAL INFORMATION ON THE FOOD PRODUCED USING GENE TECHNOLOGY **20**
Section 2.01 Nature and Identity of the Genetically Modified Food 21
(a) A description of the GM organism from which the new GM food is derived including the nature and purpose of the genetic modification 21
(b) The name, line number and OECD Unique identifier of each of the new lines or strains of GM organism from which the food is derived 21
(c) The name the food will be marketed under 21
Section 2.02 History of Use of the Host and Donor Organisms 22
(a) Common and scientific names 22
(i) Host organism 22
(ii) Donor organisms 22
a) Bacillus thuringiensis 22
b) Escherichia Coli (E.coli) strain K-12 23
c) Other donor organisms 23
Section 2.03 The Nature of the Genetic Modification 24

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(a) A description of the method used to transform maize 24
(b) A description of the construct and transformation vector used 25
(i) The size, sources, and function of all the genetic components including marker genes, regulatory and other elements 26
(ii) A detailed map of the location and orientation of all the genetic components contained within the construct and vector 26
(c) A full molecular characterisation of the genetic modification in the new organism 30
(i) Identification of all transferred genetic material and whether it has undergone any rearrangements including full DNA insert and flanking sequence analysis 30
a) Insert and flanking sequence analysis 30
b) Genomic insertion site sequence analysis 31
c) Assessment to ascertain if the MZIR260 inserts disrupts endogenous maize genes 34
d) Determination of the chromosomal location of the transgenic locus 35
(ii) A determination of the number of insertion sites, and the number of copies at each insertion site using whole genome sequencing 37
a) Presence or absence of pSYN24795 plasmid backbone sequence 37
b) T-DNA insertion site(s) and insert copy number 38
c) Gene stability 39
(iii) Map(s) illustrating the organisation of the inserted genetic material in planta 42
(iv) Analysis for occurrence of open reading frames (ORFs) in insert and junction regions 43
a) Start-to-stop ORFs spanning the MZIR260 Insert - Junction Region 44
b) Start-to-stop ORFs spanning the MZIR260 Insert Region 44
(d) A family tree and/or description of the breeding process including which generation have been used for each study 47
(e) Stability of the genetic changes 49
(i) WGS to demonstrate gene stability in T_1, T_3 and F_1 MZIR260 maize generations 49
(ii) Mendelian inheritance to demonstrate pattern of inheritance in three segregating generations of Maize - BC_2F_1, BC_3F_1, and F_2 49
(iii) ELISA to demonstrate phenotype stability in BC_2F_1, BC_4F_1 and BC_5F_1 MZIR260 maize generations 51
(f) Conclusion – Molecular Characterisation 58
Section 2.04 Annex II References 58
Article III. PART B. CHARACTERISATION AND SAFETY ASSESSMENT OF NEWLY EXPRESSED PROTEINS (NEPs) 62

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Section 3.01 Characterization of the NEPs - eCry1Gb.1Ig and PMI 64
(a) Expression levels of eCry1Gb.1Ig and PMI proteins in the MZIR260 F_1 generation across in several tissue types, developmental stages, and field environments ...64
(b) Equivalence of the eCry1Gb.1Ig protein expressed *in planta* and a microbial system 69
(i) Functional activity of eCry1Gb.1Ig 69
(ii) Immunoreactivity and molecular weight determination of eCry1Gb.1Ig 71
(iii) Amino acid sequence of eCry1Gb.1Ig 73
(iv) Peptide mass coverage analysis of eCry1Gb.1Ig 73
(v) N-terminal and C-terminal peptide sequencing of eCry1Gb.1Ig 76
(c) Equivalence of the PMI protein expressed *in planta* and a microbial system 77
(i) Functional activity of PMI 77
(ii) Immunoreactivity and molecular weight determination of PMI 79
(iii) Amino acid sequence of PMI 81
(iv) Peptide mass coverage analysis of PMI 81
(v) N-terminal peptide sequencing of PMI 82
(d) Post-translational glycosylation of microbially- and plant-produced produced Cry1Gb.1Ig and PMI proteins 83
(i) Glycosylation analysis of eCry1Gb.1Ig 83
(ii) Glycosylation analysis of PMI 85
(e) Where any ORFs have been identified, bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs. 86
(f) Conclusion - Characterisation of the NEPs 86
Section 3.02 Safety Assessment of Introduced Proteins Where the Protein is Identical to a Protein Previously Assessed by FSANZ – PMI 87
(a) Information on the potential toxicity and allergenicity of PMI 87
(i) Source organism 87
(ii) History of safe use (HOSU) in food 87
(iii) Bioinformatic comparisons of the amino acid sequence with potential toxins ..88
(iv) Bioinformatic comparisons of the amino acid sequence with potential allergens 89
(b) Conclusion on PMI safety 90
Section 3.03 Safety Assessment (Full) of Introduced Proteins Where the Protein is NOT Identical to a Protein Previously Assessed by FSANZ - eCry1Gb.1Ig 91
(a) Information on the potential toxicity and allergenicity of eCry1Gb.1Ig 91
(i) Source of the protein 91

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(ii) History of safe use (HOSU) in food 91

(iii) A bioinformatic comparison of the amino acid sequence of eCry1Gb.1Ig to known protein toxins 91

(iv) A bioinformatic comparison of the amino acid sequence of eCry1Gb.1Ig to known protein allergens 93

(v) Structural stability of the protein to enzymes found in gastrointestinal environments, heat and/or acid 94

a) In Vitro Digestibility of Microbially Produced ECRY1GB.1IG Protein under Simulated Mammalian Gastric Conditions 94

b) Effect of Temperature on the Bioactivity of eCry1Gb.1Ig Protein 98

(vi) Specific serum screening where the protein is derived from a source known to be allergenic or has sequence homology with a known allergen 99

(vii) Role of the protein, if any, in the elicitation of gluten-sensitive enteropathy, in cases where the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains. 99

(b) Conclusions on potential toxicity and allergenicity of the eCry1Gb.1Ig protein 99

Section 3.04 Novel Herbicide Metabolites in GM Herbicide - Tolerant Plants 100

Section 3.05 Compositional Analyses 101

(i) Summary of results 103

(ii) Detailed results 104

a) Forage - Proximates and fiber 104

b) Forage - Calcium and phosphorus 104

c) Grain - Proximates, fiber, and starch 104

d) Grain - Minerals 104

e) Grain - Vitamins 104

f) Grain - Amino acids 105

g) Grain - Fatty acids 105

h) Grain - Secondary metabolites and anti-nutrients 105

Section 3.06 Annex III References 121

Article IV. APPENDICES (Company Data to Support this Application) 123

Lepidopteran-Protected Maize. Syngenta Australia Pty Ltd. All Rights Reserved. 2024

LIST OF TABLES

TABLE 1. Genetic Elements in the MZIR260 Insert 27

TABLE 2. Genetic Elements in the pSYN24795 Backbone Vector 28

TABLE 3 Results of BLASTN Analysis of 1000 bp of Maize Genomic Sequence Flanking the 5' Region of the MZIR260 Insert Using the Viridiplantae EST Database 35

TABLE 4 B73 Maize Chromosome Regions with Similarity to MZIR260 Genomic Insertion Site 36

TABLE 5. Length and Coverage of Junction Sequences 41

TABLE 6. Open Reading Frames Derived from MZIR260 Insert 45

TABLE 7. Pedigree /Generation Used in Each Study 48

TABLE 8. Observed and Expected Frequencies of *eCry1Gb.1Ig-03* and *pmi-15* in the BC_2F1, BC_3F1, and F_2 Generations of MZIR260 Maize 51

TABLE 9. Concentrations of eCry1Gb.1Ig Protein in R6 and R6 Senescence Kernels and from R4 forage from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis 53

TABLE 10. Concentrations of PMI Protein in R6 and R6 Senescence Kernels and from R4 forage from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis 54

TABLE 11. Concentrations of eCry1Gb.1Ig and PMI Protein in Leaves from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis 55

TABLE 12. Concentrations of eCry1Gb.1Ig and PMI Protein in Roots from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis 56

TABLE 13. Concentrations of eCry1Gb.1Ig and PMI Protein in R1 Pollen from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis 57

TABLE 14. Concentrations of eCry1Gb.1Ig and PMI in Kernels and Forage of MZIR260 Grown Across All Locations and Growth Stages on a Dry-Weight and a Fresh-Weight Basis 66

TABLE 15. Concentrations of eCry1Gb.1Ig in Leaves, Root, Whole Plants and Pollen of MZIR260 Grown Across All Locations and Growth Stages on a Dry-Weight and a Fresh-Weight Basis 67

TABLE 16. Concentrations of PMI in Leaves, Root, Whole Plants and Pollen of MZIR260 Grown Across All Locations and Growth Stages on a Dry-Weight and a Fresh-Weight Basis 68

TABLE 17. Estimated LC_{50} for eCry1Gb.1Ig in MZIR260 Maize Crude Leaf Extract, Microbially Produced eCry1Gb.1Ig, and Nontransgenic Maize Crude Extract Fortified with Microbially-produced eCry1Gb.1Ig (168 Hours) 71

TABLE 18. Specific Enzymatic Activity of The Microbially-Produced and Plant Produced PMI Proteins 78

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TABLE 19. Syngenta Maize Applications Approved by FSANZ Containing the Identical PMI protein as found in MZIR260 87

TABLE 20. Comparison of Insecticidal Activities (120 hours) of the eCry1Gb.1Ig Samples After Temperature Treatments (25°C, 37°C, 65°C, and 95°C) with that of the Baseline Control Samples (4°C) 98

TABLE 21. List of Components Analyzed in Maize Forage and Grain 103

TABLE 22. Forage Components: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 106

TABLE 23 Grain Proximates, Fiber, and Starch Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 108

TABLE 24 Grain Mineral Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 110

TABLE 25 Grain Vitamin Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 112

TABLE 26 Grain Amino Acid Composition [Alanine–Leucine]: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 114

TABLE 27 Grain Amino Acid Composition [Lysine–Valine]: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 116

TABLE 28. Grain Fatty Acid Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 118

TABLE 29. Grain Secondary Metabolite and Anti-Nutrient Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control) 120

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LIST OF FIGURES

FIGURE 1. Maize Transformation Process for the Generation of Event MZIR260 25
FIGURE 2. Map of SYN24795 with location and orientation of all genetic elements.....29
FIGURE 3. Location of the Primers Used for Amplification of the Genomic Insertion Site of the MZIR260 Insert 32
FIGURE 4. Process Used to Determine the Genomic Insertion Site of the MZIR260 Insert and to Assess the Changes in the Insertion Site.....33
FIGURE 5. Alignment of the Consensus Sequences of Individual PCR Clones and Final Consensus Sequence of the Genomic Insertion Site Where MZIR260 is Inserted in the AX5707 Maize Genome 33
FIGURE 6. Nucleotide Sequence of the Genomic Insertion Site Where MZIR260 is Inserted in the AX5707 Maize Genome 33
FIGURE 7. Alignment of the Genomic Insertion Site Sequence from Nontransgenic, near-isogenic AX5707 Maize to the 1000-bp 5' and 1000-bp 3' Genomic Sequences Flanking the MZIR260 Insert 33
FIGURE 8. Representation of MZIR260 Genomic Insertion Site 34
FIGURE 9. Region of B73 Maize Chromosome 2 with Highest Similarity to MZIR260 Genomic Insertion Site 36
FIGURE 10. Flowchart of the Bioinformatic Analysis Using the ISC Method.....39
FIGURE 11. Junction Sequence Analyses and Insertion Site of Event MZIR260 over Three (3) Generations 41
FIGURE 12. Organisation of the inserted genetic materials *in planta:* T-DNA from pSYN24795 transferred to MZIR260 42
FIGURE 13. Pedigree Breeding of the MZIR260 Maize.....47
FIGURE 14. Locations of the Real-time qPCR Primers and Probes in the pSYN24795 Plasmid T-DNA 50
FIGURE 15. Western Blot Analysis of the Microbially Produced and Plant-Produced eCry1Gb.1Ig 72
FIGURE 16. Amino Acid (Deduced) Sequence of the eCry1Gb.1Ig Protein.....73
FIGURE 17. Amino Acid Sequence Coverage Map for Microbially-produced eCry1Gb.1Ig.....75
FIGURE 18. Amino Acid Sequence Coverage Map for Plant-produced eCry1Gb.1Ig.....75
FIGURE 19. Amino Acid Sequence Analysis of N-Terminal Peptides of Microbially-produced and Plant-produced eCry1Gb.1Ig Proteins 76
FIGURE 20. Amino Acid Sequence Analysis of C-Terminal Peptides of Microbially produced and Plant-produced eCry1Gb.1Ig Proteins 76
FIGURE 21. Western Blot Analysis of the Microbially-Produced and Plant-Produced PMI Proteins 80
FIGURE 22. Amino acid (Deduced) Sequence of the PMI Protein.....81

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FIGURE 23. Amino Acid Sequence Coverage Map for Microbially-Produced PMI Protein 81
FIGURE 24. Amino Acid Sequence Coverage Map for Purified Plant-Produced PMI Protein 82
FIGURE 25. Amino Acid Sequence Analysis of N-terminal Peptides of Microbially Produced and Purified Plant-Produced PMI Proteins 83
FIGURE 26. Glycosylation Analysis of the Microbially-Produced and Plant-Produced eCry1Gb.1Ig 84
FIGURE 27. Glycosylation Analysis of the Microbially-Produced and Plant-Produced PMI 85
FIGURE 28. SDS-PAGE Analysis of eCry1Gb.1Ig Following Digestion in SGF 96
FIGURE 29. Western Blot Analysis of eCry1Gb.1Ig Following Digestion in SGF 97
FIGURE 30. Map Trial Locations 102

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PART 1 GENERAL REQUIREMENTS

1.1 Applicants and Developers Details

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Developer:
Syngenta Crop Protection, LLC - part of the Syngenta Group, a leading agriculture company helping to improve global food security.

1.2 Purpose of the Application

All foods sold in Australia and New Zealand (ANZ) must comply with food standards. These standards are compiled in the Australia New Zealand Food Standards Code. Foods produced using gene technology require pre-market clearance and to be listed in Schedule 26 of the Food Standards Code.

Syngenta is seeking to amend the Australia New Zealand Food Standards Code to permit the sale and use of food derived from a new food produced using gene technology: Event MZIR260 Maize, under Standard 1.5.3 This maize has been genetically modified for protection from lepidopteran insect pests primarily FAW, *Spodoptera frugiperda*.

1.3 Justification of the Application

a) Need for the Proposed Change

Syngenta has developed a new GM Event MZIR260 maize. Cultivation of MZIR260 maize is targeted primarily to Brazil and Argentina. Event MZIR260 maize is planned to be marketed in stack combinations with other GM traits.

In 2022, Australia and New Zealand (ANZ) produced a combined amount of just over 0.5 million tonnes of maize used mainly for domestic supply. Domestic supply of maize products is supplemented by overseas imports. These may include, but not limited to starch, grits, meal, flour, oil, and sweetener products. Event MZIR260 maize is likely to enter Australia and New Zealand as food derived from Stacked Event Crops containing Event MZIR260. Pre-market approval is necessary before GM foods can enter the Australian and New Zealand food supply.

b) Advantage of the GMO

Insect pests cause immense agronomic losses worldwide. One of the most destructive pests of maize crops is (FAW, *Spodoptera frugiperda*). Syngenta has developed a maize line with improved insecticidal activity against (FAW, *Spodoptera frugiperda*). This maize line,

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designated Event MZIR260, was developed to provide a method to prevent yield losses from feeding damage caused by (FAW, *Spodoptera frugiperda*) larvae. Insecticidal proteins, for example those produced by the bacterium *Bacillus thuringiensis* (*B. thuringiensis* or *Bt*), are among the safest and most effective insect control agents. GM crops expressing such proteins are a key part of a successful integrated pest management (IPM) program for (FAW, *Spodoptera frugiperda*).

1.4 Regulatory Impact Information

Crops in particular maize improved through modern biotechnology have shown significant benefits for agriculture by offering tools for farmers to better manager weeds, pests, and disease, and ultimately maximising on opportunities to improve crop quality and yields. Based on a USDA survey, Bt corn acreage in the United States (US) grew from approximately 8 percent in 1997 to 19 percent in 2000, before climbing to 85 percent in 2023 (USDA ERS 2003).

In 2022/2023 maize (field corn) remains as the number one produced cereal crop worldwide with an estimated 1.2 billion tonnes produced (USDA FAS, 2024). In 2022/2023, the top producer of corn was the US with 335.7 million tonnes (~ 30% of global production), followed by China which produced 277.2 million tonnes (~20% of global production). In 2022, North America was the largest exporter of viable and processed corn globally, predominantly yellow dent field corn (HIS Markit, 2023)

Two regulatory options exist, either (1) no approval; or (2) approval of food from MZIR260 maize based on the conclusions of this safety assessment.

1.5 Costs, Benefits, and Impact on Trade

The costs, benefits and impact on trade are the same as those described in previous corn applications submitted to FSANZ by Syngenta (A385; A386; A564; A580; A1001; A1060; A1112; A1116).

1.6 Exclusive Capturable Commercial Benefits (ECCB)

Exclusive Capturable Commercial Benefit (ECCB) is applicable. Syngenta accepts the full costs associated with this application, which is expected to fall within the General Procedure Category.

1.7 Confidential Commercial Information

Syngenta requests Food Standards Australia and New Zealand, to ensure information supplied in this application clearly marked as ‘Confidential Business Information’ (CBI), remains confidential and is not shared with third party stakeholders.

1.8 International and Other Standards

Syngenta reports and studies included in the information supporting this application have been conducted according to international standards. In the safety assessment of biotechnology

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products, Syngenta referred primarily to the *Codex Alimentarius* Commission Foods Derived from Modern Biotechnology (CAC 2009), and the relevant Codex Standard is as follows:

- Principles for the risk analysis of foods derived from modern biotechnology (CAC/GL 44-2003)
- Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants (CAC/GL 45-2003)

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1.9 Statutory Declaration

Commonwealth of Australia
STATUTORY DECLARATION
Statutory Declarations Act 1959

I, Rhonda Hall, of Level 1, 2-4 Lyonpark Road, Macquarie Park NSW 2113 , Regulatory Manager, make the following declaration under the Statutory Declarations Act 1959:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the Statutory Declarations Act 1959, and I believe that the statements in this declaration are true in every particular.

I believe that the statements in this declaration are true in every particular, and I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the Statutory Declarations Act 1959, the punishment for which is imprisonment for a term of 4 years.

Signature

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myGov

This Commonwealth statutory declaration was executed using myGov, an online platform prescribed for that purpose under section 9A of the Statutory Declarations Act 1959.

Declared on 06 May 2024 at 02:05PM +1000

Declaration made by Rhonda Hall at 2/46 Fourth Avenue Palm Beach QLD 4221

The declarant's identity was verified via an approved identity service to make this statutory declaration for the purposes of section 9A of the Statutory Declarations Act 1959.

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Page 1 of 1

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1.10 Checklist

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory requirements**
✓		**A** Form of application ✓ *Application in English* ✓ *Executive Summary (separated from main application electronically)* ✓ *Relevant sections of Part 3 clearly identified.* ✓ *Pages sequentially numbered.* ✓ *Electronic copy (searchable)* ✓ *All references provided*
✓	Page 13	**B** Applicant details
✓	Page 13	**C** Purpose of the application
✓	Page 13	**D** Justification for the application ✓ *Regulatory impact information* ✓ *Impact on international trade*
✓	Page 14	**E** Information to support the application ✓ *Data requirements*
✓		**F** Assessment procedure ✓ *General* ☐ *Major* ☐ *Minor* ☐ *High level health claim variation*
✓		**G** Confidential commercial information ✓ *CCI material separated from other application material.* ✓ *Formal request including reasons.* ✓ *Non-confidential summary provided*
✓		**H** Other confidential information ✓ *Confidential material separated from other application material.* ✓ *Formal request including reasons*
✓	Page 14	**I** Exclusive Capturable Commercial Benefit ✓ *Justification provided*
✓	Page 14	**J** International and other national standards ✓ *International standards* ✓ *Other national standards*
✓	Page 16	**K** Statutory Declaration ✓
✓	Pages 17-18	**L** Checklist/s provided with application ✓ *3.1.1 Checklist* ✓ *All page number references from application included.* ✓ *Any other relevant checklists for Chapters 3.2–3.7*

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Foods produced using gene technology (3.5.1)		
Check	**Page No.**	**Mandatory requirements**
✓	Page: 21	**A.1** Nature and identity of the genetically modified food
✓	Pages: 22-23	**A.2** History of use of host and donor organisms
✓	Pages: 24-61	**A.3** Nature of genetic modification
✓	Pages: 61-99	**B.1** Characterisation and safety assessment
✓	Pages: 61-99 PMI Pages: 87-90 eCry1Gb.1Ig-03 Pages: 91-99	**B.2** New proteins (NEPs)
✓	Not applicable	**B.3** Other (non-protein) new substances If other (non-protein) substances are produced as a result of the introduced DNA
✓	Not applicable	**B.4** Novel herbicide metabolites in GM herbicide-tolerant plants
✓	Pages: 101-120	**B.5** Compositional analyses
✓	Not applicable	**C** Nutritional impact of GM food
✓	Not applicable	**D** Other information

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Article II. PART A. TECHNICAL INFORMATION ON THE FOOD PRODUCED USING GENE TECHNOLOGY

Article II - Section 2.01 describes the nature, identity, and purpose of the Genetically Modified Organism (GMO) including the OECD Unique identifier.

Article II - Section 2.02 describes the history of use of the host and donor organisms used to create the GMO, and for most part for this application, only the common and scientific names have been included. This is because, where information relating to an organism has been included in previous safety assessments prepared by FSANZ, it is not necessary to provide any further information other than the common and scientific names of host and donor organisms which according to the FSANZ handbook must always be stated.

Therefore, information can be relied on from previous assessments. This includes for the host organism any known pathogenicity, toxicity or allergenicity of relevance to the food; history of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant); the part of the organism typically used as food the types of products likely to include the food or food ingredient and whether special processing is required to render food derived from the organism safe to eat.

For the donor organism(s) this includes, any known pathogenicity, toxicity or allergenicity of relevance to the food and history of use of the organism in the food supply or history of human exposure to the organism through means other than intended food use (e.g. as a normal contaminant).

Article II - Section 2.03 provides an understanding of the DNA introduced into the host genome and helps to inform the safety assessment in relation to both the intended and possible unintended effects resulting from the transformation (OECD, 2010).

The transformation method together with a detailed description of any DNA sequences that were transferred to the host genome and a breeding pedigree is provided.

Furthermore, information is supplied to describe the configuration of genetic elements introduced into the host organism; the nature and number of expression cassettes and the number of insertion sites, including a description of any rearrangements or deletions that may have occurred as a result of the transformation.

This section also includes the identification and analysis of any unintended open reading frames (ORFs) of significant length created as a result of the insertion event. The stability of the genetic modification at both the genotypic and phenotypic level is shown including Mendelian analysis. The new genetic material is considered a stable part of the host genome if they remain the same over several generations of plants produced by conventional breeding. Analysis of inheritance can show whether the inserted DNA has been stably integrated into the host genome and inherited from one generation to the next following Mendelian principles. This provides assurance that the safety assessment is applicable to future generations.

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Section 2.01 Nature and Identity of the Genetically Modified Food

(a) A description of the GM organism from which the new GM food is derived including the nature and purpose of the genetic modification

Maize (*Zea mays* L.) has been genetically modified to produce Event MZIR260 maize, which provides control of fall armyworm (FAW) (*Spodoptera frugiperda*). Maize plants derived from transformation Event MZIR260 produce the insecticidal protein eCry1Gb.1Ig encoded by the gene *eCry1Gb.1Ig-03*. The engineered protein eCry1Gb.1Ig is a chimera of Cry1Gb and Cry1Ig. Both Cry1Gb and Cry1Ig are active against several lepidopteran pest species and were derived from sequenced genomes of the soil bacterium *Bacillus thuringiensis*. The protein eCry1Gb.1Ig was engineered to have improved insecticidal activity against (FAW) (*Spodoptera frugiperda*). MZIR260 maize plants also produce the enzyme phosphomannose isomerase (PMI), encoded by the gene *pmi-15* (also known as *manA*) derived from *Escherichia coli* strain K-12. PMI enables transformed plant cells to utilize mannose as a primary carbon source; it was used as a selectable marker in the development of MZIR260 maize.

(b) The name, line number and OECD Unique identifier of each of the new lines or strains of GM organism from which the food is derived

The name of the GM organism is Event 'MZIR260' maize, with the assigned unique identifier OECD ID: SYN-ØØ26Ø-3. Common names include, MZIR260 maize; MZIR260 corn; Event MZIR260 maize; Event MZIR260 corn; MZIR260.

(c) The name the food will be marketed under

A commercial trade name has not yet been decided. There are no plans to cultivate this product in Australia or New Zealand.

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Section 2.02 History of Use of the Host and Donor Organisms

(a) Common and scientific names

(i) Host organism

The host organism is *Zea mays* L (maize, corn) and the recipient line used in the plant transformation is 'AX5707'.

Information relating to 'Maize' as the host organism in a GM variety can be found in at least 30 safety assessments prepared by FSANZ. Of those, 8 were submitted by Syngenta for evaluation (A385; A386; A564; A580; A1001; A1060; A1112; A1116).

Maize is a large annual monoecious grass that is grown for animal feed, silage, human grain, vegetable oil, sugar syrups, and other miscellaneous uses. In 2024, maize continues to be a fundamental food source for humans globally and as a feed source for various livestock. It does not contain significant toxins or allergens, showcasing a robust track record of safe use as a food crop. Maize contains low levels of some anti-nutrients, such as phytic acid, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), and raffinose. Maize also contains low levels of trypsin and chymotrypsin inhibitors, neither of which is considered nutritionally significant (OECD 2002).

- Scientific name: *Zea mays* L.
- Common names: Maize, corn
- *Zea* is a genus of the family Gramineae (Poaceae), commonly known as the grass family (Gould, and Shaw, 1983)
- The genus consists of some four species: *Z. mays*, *Z. diploperennis*; *Z. luxurians* and *Z. perennis*. Variations of the species have been assigned to the segregate genus *Euchlaena*, which is not currently recognized, or have been divided into numerous small species within the genus *Zea* (Terrell, 1986)
- Of the four species, only *Z. mays* is abundantly cultivated world-wide. The other species are occasional university or experiment station research subjects.

(ii) Donor organisms

The donor organisms of the transgenes are *Bacillus thuringiensis* and *Escherichia Coli* strain K-12.

a) Bacillus thuringiensis

The gene *eCry1Gb.1Ig* encodes a chimeric protein that was engineered by swapping domains of two Cry proteins, Cry1Gb and Cry1Ig protein (Chae *et al.* 2022). Both parental proteins were sourced from the soil bacterium *Bacillus thuringiensis*, commonly known as *Bt.* Insecticidal proteins, produced by the bacterium *Bt* are considered among the safest insect control agents. Many of the GM crops approved for commercial use to control insect pests are cry proteins derived from *Bt*.

- Scientific name: *Bacillus thuringiensis*
- Common name: *Bt; B.t*
- *Bacillus thuringiensis* belongs to the *Bacillus cereus group*, *Bacillus* genus, and Bacillaceae family (Schoch *et al.* 2020).

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Bt is the source of transgenes expressed in GM foods previously assessed and approved by FSANZ in several applications submitted by Syngenta including: *cry1Ab* gene (A385/A386); *cry1Ab* gene (A385/A386); *mcry3A (*A564/A1116); *vip3Aa* gene (A1001); *ecry3.1Ab* - engineered from selected portions of the *mCry3A* and *Cry1Ab* genes (A1060/ A1116).

b) Escherichia Coli (E.coli) strain K-12

Escherichia Coli strain K-12 is the source of the *pmi* gene. *E. coli* K-12 is considered a well characterised strain of *E. coli.* and non-pathogenic. It is not considered to be toxic (US EPA, 2007), allergenic or pathogenic in mammals (Taylor and Hefle 2001).

The *pmi* gene from *E. coli* K-12 has been used as a selective marker in numerous commercialized transgenic crops since 2006 (ISSAA, 2024). The food and feed safety of genetically modified crops expressing the *pmi* gene has been extensively evaluated by regulatory agencies worldwide, which also demonstrates the safety of *E. coli* K-12 being used as a source organism.

- Scientific name: *Escherichia Coli* strain K-12 (Taxonomy ID 83333)
- Common name: non-pathogenic *E.coli*; *E.coli* K-12
- *E. coli* K-12 belongs to the genus *Escherichia*, family Enterobacteriaceae, order Enterobacterales, and class Gammaproteobacteria.

FSANZ has assessed and approved several applications submitted by Syngenta with the *pmi* gene derived from *E. coli* K-12, including: A564 (Event MIR604); A580 (Event 3272 maize); A1001 (Event MIR162 maize); A1060 (Event 5307 maize).

c) Other donor organisms

The source of all genetic elements is summarised in Table 1 and 2.

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Section 2.03 The Nature of the Genetic Modification

(a) A description of the method used to transform maize

Transformation of *Zea mays* to produce Event MZIR260 maize was accomplished using *Agrobacterium tumefaciens*-mediated transformation of immature embryos, as described in Zhong *et al*. (2018) and summarized in Figure 1. Using this method, the genetic elements within the left and right border regions of the transformation plasmid were efficiently transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions were not transferred.

Agrobacterium strain LBA4404 (*recA*⁻) harboring the binary transformation plasmid pSYN24795 was prepared for its use in maize transformation following the methods described in Li *et al*. (2003). The *recA*⁻ version of LBA4404 was used to maximize plant transformation frequencies and is often used with binary vectors that are known to have repetitive sequence or large transferred DNA (T-DNA) inserts. Immature embryos from greenhouse-grown Syngenta's elite inbred line AX5707 were harvested approximately 9 days after pollination and used as explants (Zhong *et al*. 2018). Immature embryo isolation, *Agrobacterium* inoculation and co-cultivation of *Agrobacterium* with the immature embryos were performed as described in Zhong *et al*. (2018) using the bulk extraction method described therein (Figure 1).

After initial incubation with *A. tumefaciens*, the transformed tissue was transferred to selective media containing the broad-spectrum antibiotic timentin, thus ensuring that the *A. tumefaciens* was cleared from the transformed tissue. Timentin has been shown to efficiently eliminate *A. tumefaciens (*Nauerby *et al.,* 1997*)* and was used throughout the *in vitro* selection process. In addition, the phosphomannose isomerase (PMI) gene was used as a selectable marker (Negrotto *et al*., 2000) and transformed tissues and putative transgenic events were regenerated and rooted as previously described (Zhong *et al*. 2018) using media amended with mannose. The transformation system with PMI marker gene and mannose as a selection agent has been optimized for the generation of transgenic events using AX5707. Mannose-resistant embryogenic calli were transferred to plant regeneration medium and putative transgenic shoots at least 2 cm in length were transferred to rooting medium. Approximately 14 days after culture on rooting medium, deoxyribonucleic acid (DNA) extracted from approximately 1 cm leaf segments was screened for the presence of *eCry1Gb.1Ig* and *pmi*, and for the absence of plasmid backbone sequences by real-time polymerase chain reaction (PCR) analysis (Ingham *et al*., 2001). This screen allows for the selection of transgenic events that carry the T-DNA and are free of plasmid backbone DNA. Regenerated T_0 plants containing single copies of *eCry1Gb.1Ig* and *pmi* and negative for plasmid backbone were transferred to the greenhouse for further propagation.

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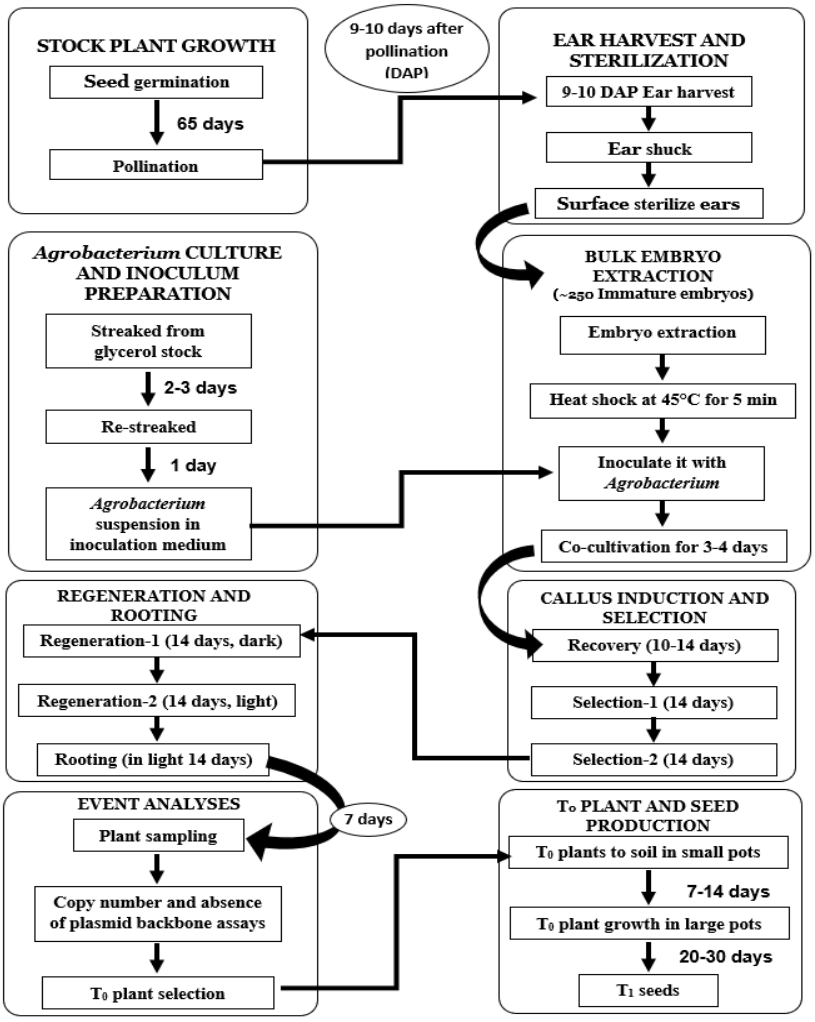

FIGURE 1. Maize Transformation Process for the Generation of Event MZIR260

(b) A description of the construct and transformation vector used

The transformation plasmid pSYN24795 used to generate Event MZIR260 contains the genes *eCry1Gb.1Ig-03* and *pmi-15*. The pSYN24795 plasmid consists of two cassettes and backbone sequences. The full details on the construction of plasmid pSYN24795, via a series of molecular manipulations are provided in the study report (Appendix 01).

Appendix 01. Herrero, Sonia. RIR-0007259. (2023). *Plasmid pSYN24795 Plasmid Lineage Analysis and Sequence. Assessment.* Research Triangle Park, NC 27709-2257 USA: Unpublished. Syngenta Crop Protection, LLC.

In summary, the *eCry1Gb.1Ig-03* cassette contains the gene *eCry1Gb.1Ig-03* regulated by a ubiquitin promoter from sugarcane (*Saccharum officinarum*) (SoUbi4-02) and a ubiquitin terminator from maize (*Zea mays* L.) (ZmUbi361-05). The engineered gene *eCry1Gb.1Ig-03* is a synthesized, codon-optimized chimera of *Cry1Gb* and *Cry1Ig* (Chae *et al.* 2022). Both Cry1Gb and Cry1Ig proteins are derived from sequenced genomes of *Bacillus thuringiensis*

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and are active against several lepidopteran pest species. The *pmi-15* cassette contains the gene *pmi-15* regulated by a ubiquitin promoter (Ubi1-43) and a ubiquitin terminator (Ubi1-04), both derived from maize. The gene *pmi-15* (also known as *manA*) was derived from *Escherichia coli* strain K-12. The protein phosphomannose isomerase (PMI) enables transformed plant cells to utilize mannose as a primary carbon source; it was used as a selectable marker in the development of MZIR260 maize.

The plasmid pSYN24795 backbone contains left border (LB-01-01) and right border (RB-01-01) sequences, and the *VirG-01* gene and promoter from *Agrobacterium tumefaciens* Ti plasmids, the aminoglycoside adenylyltransferase gene (*aadA-03*) from *Escherichia coli* transposon Tn7, the pVS1 replication protein gene (*repA-03*) from *Pseudomonas aeruginosa*, the VS1-02 origin of replication (ori) from *P. aeruginosa*, and the ColE1-06 ori from *E. coli*.

(i) The size, sources, and function of all the genetic components including marker genes, regulatory and other elements

Detailed descriptions of the genetic elements are provided in Tables 1 and 2.

(ii) A detailed map of the location and orientation of all the genetic components contained within the construct and vector

A map of pSYN24795 is provided in Figure 1 showing location and orientation of all genetic components. Since WGS was used instead of Southern Analysis to characterize the Event, a detailed map with restrictions sites is not applicable.

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TABLE 1. Genetic Elements in the MZIR260 Insert

Plasmid pSYN24795 *eCry1Gb.1Ig-03* Cassette: Genetic Elements and Descriptions			
Genetic element	**Size (bp)**	**Position in pSYN24795**	**Description**
Region-01	91	26 to 116	Region used for cloning.
SoUbi4-02 promoter[a]	1802	117 to 1918	Constitutive *Saccharum officinarum* (sugar cane) Ubiquitin 4 promoter containing the first intron (NCBI accession number AF093504.1) (Wei *et al*. 1999).
Region-02	12	1919 to 1930	Region used for cloning.
eCry1Gb.1Ig-03	3510	1931 to 5440	Sequence encoding the engineered protein eCry1Gb.1Ig (Chae *et al*. 2022), which is a chimera of Cry1Gb and Cry1Ig. Both Cry1Gb and Cry1Ig proteins are derived from *Bacillus thuringiensis* and are active against several lepidopteran pest species. The eCry1Gb.1Ig protein was engineered to have improved insecticidal activity against fall armyworm (*Spodoptera frugiperda*).
Region-03	6	5441 to 5446	Region used for cloning.
ZmUbi361-05 terminator	1001	5447 to 6447	The terminator derived from the maize Ubiquitin gene (similar to NCBI accession number U29162.1) (Nuccio *et al*. 2018).
Region-04	56	6448 to 6503	Region used for cloning.
Plasmid pSYN24795 *pmi-15* Cassette: Genetic Elements and Descriptions			
Ubi1-43 promoter	1993	6504 to 8496	Promoter region from *Zea mays* ubiquitin 1 gene containing the first intron (NCBI accession number S94464.1). Provides constitutive expression in monocots (Christensen *et al*. 1992).
Region-05	12	8497 to 8508	Region used for cloning.
pmi-15	1176	8509 to 9684	*Escherichia coli* gene *pmi* encoding the enzyme PMI (NCBI accession number M15380.1); also known as *manA* which catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate (Negrotto *et al*. 2000).
Region-06	38	9685 to 9722	Region used for cloning.
Ubi1-04 terminator	1035	9723 to 10757	The terminator from the ubiquitin 1 gene from *Z. mays* (similar to NCBI accession number S94464.1) Christensen *et al*. 1992).
Region-07	117	10758 to 10874	Region used for cloning.

[a] When compared to the transformation plasmid pSYN24795, there is a single base pair change (guanine to thymine) located in the sugarcane-derived ubiquitin promoter (SoUbi4-02). Further information can be found in (Appendix 02. Bartaula, Radhika. RIR-0007263 - Volume 1. Amendment 1, 2024), and Figure 12 of this application dossier.

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TABLE 2. Genetic Elements in the pSYN24795 Backbone Vector

Plasmid pSYN24795 Backbone: Genetic Elements and Descriptions			
Genetic element from pSYN24795	**Size (bp)**	**Position in pSYN24795**	**Description**
LB-01-01	25	10875 to 10899	Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti plasmid (NCBI accession number J01825.1). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Yadav *et al*. 1982).
Region-08	349	10900 to 11248	Region used for cloning.
aadA-03	789	11249 to 12037	Aminoglycoside adenylyltransferase gene from *E. coli* transposon Tn7 (similar to NCBI accession number X03043.1). Confers resistance to streptomycin and spectinomycin and is used as a bacterial selectable marker (Fling *et al*. 1985).
Region-09	94	12038 to 12131	Region used for cloning.
virG-01 promoter	131	12132 to 12262	Promoter region of the gene *virG* from *A. tumefaciens* composed of two promoter elements, one responsive to acetosyringone and phosphate-starvation and another to medium acidification (Winans 1990).
Region-10	74	12263 to 12336	Region used for cloning.
virG-01	726	12337 to 13062	The VirGN54D gene from pAD1289 (similar to NCBI accession number AF242881.1). The N54D substitution results in a constitutive VirG phenotype. The gene *virG* is part of the two-component regulatory system for the virulence regulon in *A. tumefaciens* (Hansen *et al*. 1994).
Region-11	29	13063 to 13091	Region used for cloning.
repA-03	1074	13092 to 14165	Gene encoding the pVS1 replication protein from *Pseudomonas aeruginosa* (similar to NCBI accession number AF133831.1), which is a part of the minimal pVS1 replicon that is functional in Gram-negative, plant-associated bacteria (Heeb *et al*. 2000).
Region-12	42	14166 to 14207	Region used for cloning.
VS1-02 ori	405	14208 to 14612	Consensus sequence for the origin of replication and partitioning region from pVS1 of *P. aeruginosa* (NCBI accession number U10487.1). Serves as the origin of replication in the *A. tumefaciens* host (Itoh *et al.* 1984).
Region-13	677	14613 to 15289	Region used for cloning.
ColE1-06 ori	807	15290 to 16096	Origin of replication (similar to NCBI accession number V00268.1) that permits replication of plasmids in *E. coli* (Itoh and Tomizawa 1979).
Region-14	112	16097 to 16208	Region used for cloning.
RB-01-01	25	1 to 25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti plasmid (NCBI accession number J01826.1). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*. 1984).

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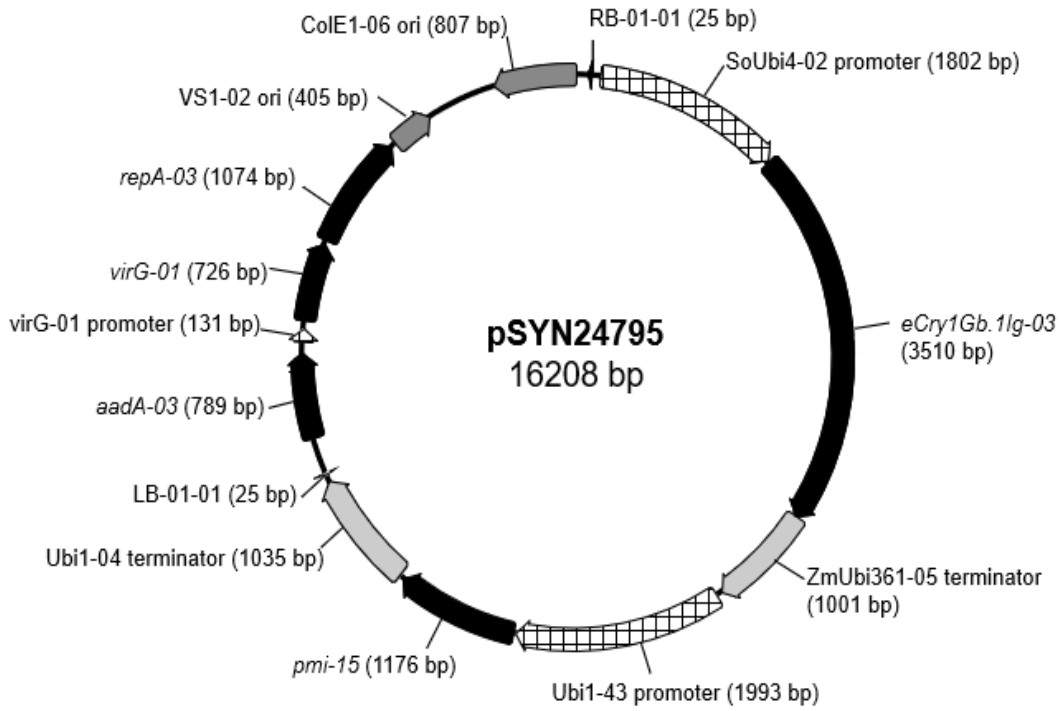

FIGURE 2. Map of pSYN24795 with location and orientation of all genetic elements

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(c) A full molecular characterisation of the genetic modification in the new organism

To ensure no unintended changes occurred to the maize genome during the development of the MZIR260 maize, detailed characterizations were conducted at the molecular level, including confirmation of the T-DNA insertion site, integrity of the insert, copy number, and absence of integration of plasmid backbone sequences. Additionally, stability of the transgenes was confirmed at both the gene and protein level across multiple generations.

(i) Identification of all transferred genetic material and whether it has undergone any rearrangements including full DNA insert and flanking sequence analysis

a) Insert and flanking sequence analysis

A study describing the insert and flanking sequence analysis, is included as part of this submission dossier (Appendix 02 and 03).

Appendix 02. Bartaula, Radhika. RIR-0007263 - Volume 1. Amendment 1. (2024). *Event MZIR260 Maize: Insert and Flanking Sequence Analysis. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

Appendix 03. Bartaula, Radhika. RIR-0007263 - Volume 2. Amendment 1.CBI. (2024). *Event MZIR260 Maize: Insert and Flanking Sequence Analysis. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

The deoxyribonucleic acid (DNA) sequence of the MZIR260 insert and 1000 base pairs (bp) of the maize genomic regions flanking the insert was determined to assess the intactness of the insert, the organization of the functional elements, and the presence of any rearrangements (e.g., deletions, insertions, inversions, duplications, and translocations) and/or base pair changes within the MZIR260 insert. The MZIR260 insert sequence was compared to the transferred DNA (T-DNA) sequence in the MZIR260 transformation plasmid pSYN24975 to determine if any changes had occurred during T-DNA integration.

In summary, etiolated MZIR260 T_3 maize plants were used to extract high molecular weight (HMW) genomic DNA (gDNA) from isolated nuclei. The HMW gDNA was used to construct a fosmid library which was subsequently characterised. Library sizing was determined from randomly selected fosmid clones that contained Event MZIR260 and the average insert size was ~38 kilobase pairs. Based on a total of 922,560 MZIR260 fosmid clones, the genomic coverage was estimated to be approximately 13X. The fosmid library was screened by PCR analyses and fosmid DNA from positive clones was isolated. The fosmid DNA from two positive clones was verified for the presence of the Event MZIR260 by restriction enzyme analysis.

The fosmid DNA from two positive clones was sequenced in a PacBio® Sequel II® platform using PacBio® Single Molecule Real-Time (SMRT®) sequencing technology. PacBio® Circular Consensus Sequencing (CCS) reads, which are consensus sequences derived from subreads generated from a single polymerase read, were analyzed using a computational analysis pipeline.

The average per-base coverage for the two fosmid clones was 106704X and 89206X. For each fosmid clone, CCS reads were mapped to a reference sequence containing the MZIR260 insert,

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and maize genomic flanking sequences and results were used to generate a consensus sequence for the MZIR260 insert and genome flanking regions in MZIR260 maize. Variant analysis identified a putative variant within a homopolymer region (13 cytosines) located in the maize-derived ubiquitin promoter in the MZIR260 insert. This was checked by Sanger sequencing and the results confirmed the absence of variants within the homopolymer region in both clones.

In Conclusion, comparison of the MZIR260 insert and flanking final consensus sequence to the transformation plasmid pSYN24795 confirmed:

- The MZIR260 insert is intact with no rearrangements.
- A single base pair change within the intron of a sugarcane-derived ubiquitin promoter (from Guanine to Thymine at position 1786) was found in the MZIR260 insert.
- Truncations were observed at the right and left border sequences of the pSYN24795 T-DNA during genomic integration of T-DNA including the entire right border along with 16 bp of T-DNA derived intervening sequence, and 8 bp of the 25 bp left border were truncated. However, since these deletions are located outside of the expression cassettes found in the MZIR260 insert, they do not affect the functionality of the contained elements.

b) Genomic insertion site sequence analysis

A study to assess the potential changes that may have occurred at the genomic insertion site during integration, is included as part of this submission dossier (Appendix 04 and 05).

Appendix 04. Kandel, Prem. RIR-0007265 - Volume 1. (2024). *Event MZIR260 Maize: Genomic Insertion Site Analysis. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

Appendix 05. Kandel, Prem. RIR-0007265 - Volume 2 CBI. (2024). *Event MZIR260 Maize: Genomic Insertion Site Analysis. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

In summary, to determine the effects of the T-DNA insertion on the host genome, the maize genomic sequence at the point of integration of the MZIR260 insert was determined using polymerase chain reaction (PCR) and Sanger sequencing, and compared to the non-transgenic, near-isogenic AX5707 maize genome. The analysis revealed the deletion of a contiguous 30 base-pairs DNA sequence of the maize genome at the insertion site where the MZIR260 insert integrated into the AX5707 maize genome.

Essentially, the genomic insertion site of the MZIR260 insert in the maize genome was determined by sequencing nontransgenic AX5707 maize, a near-isogenic line to MZIR260 maize and comparing it with the 5' and 3' genomic flanking sequence from the MZIR260 insert in Event MZIR260 previously determined by Radhika, 2024 (*unpublished*) - Appendix 02.

The MZIR260 genomic insertion site was first amplified from the nontransgenic, near-isogenic AX5707 maize control material using PCR. The PCR reactions were performed in triplicate and were individually run on an agarose gel, after which fragments of the expected size were excised, purified, and cloned into a commercially available vector. From each cloning reaction, three colonies were randomly selected, and plasmid DNA was prepared. After confirmation

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of positive clones by restriction digestion and end-sequencing, the insert sequence in one selected clone for each PCR reaction was fully determined by Sanger sequencing using primers spanning the PCR amplicon. Resulting Sanger reads were trimmed and assembled to generate consensus sequence for each PCR clone. The three consensus sequences were aligned to generate the final consensus sequence. This genomic insertion site sequence from nontransgenic AX5707 maize was then aligned to the 1000-base pairs (bp) 5' and 1000-bp 3' genomic sequences flanking the MZIR260 insert in MZIR260 maize.

- Figure 3 shows the location of the primers used for amplication of the genomic insertion site.
- Figure 4 shows the process used to determine the Genomic Insertion Site of the MZIR260 Insert.
- Figure 5, 6 and 7 redirects the reader to the study report containing confidential business information (Appendix 05).
- Figure 8 shows a map depicting the organization of the insert in the maize genome.

The alignment of three consensus sequences of the individual PCR clones is presented in Figure 5, and the MZIR260 genomic insertion site sequence in nontransgenic, near-isogenic AX5707 maize generated in this study is presented in Figure 6. Alignment between the genomic insertion site sequence and the 5′ 1000-bp and 3′ 1000-bp flanking sequences of Event MZIR260 is presented in Figure 7. This alignment showed that a contiguous 30-bp DNA sequence in the AX5707 maize genome was deleted from the insertion site during integration of the MZIR260 insert.

In Conclusion, sequence analysis of the MZIR260 genomic insertion site confirmed:

- Deletion of a contiguous 30-bp region from the native maize genomic sequence when the MZIR260 insert integrated into the maize genome.

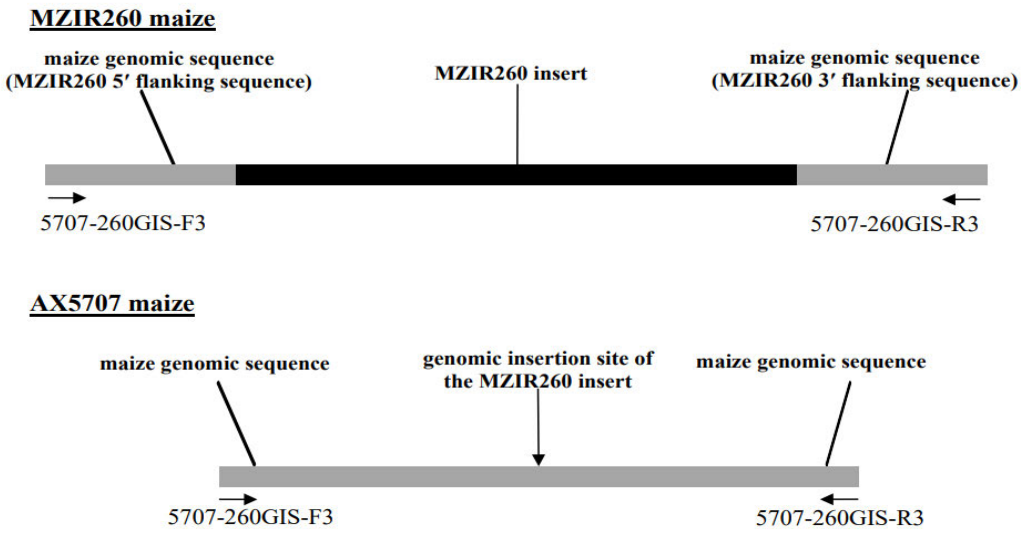

FIGURE 3. Location of the Primers Used for Amplification of the Genomic Insertion Site of the MZIR260 Insert

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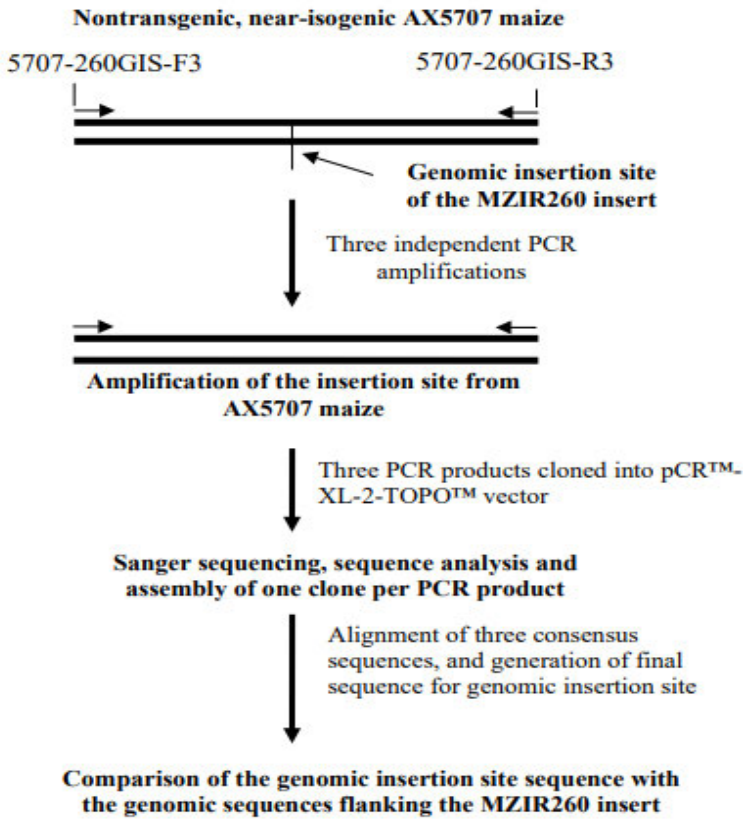

FIGURE 4. Process Used to Determine the Genomic Insertion Site of the MZIR260 Insert and to Assess the Changes in the Insertion Site

{Refer to Figure 3 (Appendix 05. Kandel, Prem. RIR-0007265 - Volume 2 CBI, 2024)}

FIGURE 5. Alignment of the Consensus Sequences of Individual PCR Clones and Final Consensus Sequence of the Genomic Insertion Site Where MZIR260 is Inserted in the AX5707 Maize Genome

{Refer to Figure 4 (Appendix 05. Kandel, Prem. RIR-0007265 - Volume 2 CBI, 2024) }

FIGURE 6. Nucleotide Sequence of the Genomic Insertion Site Where MZIR260 is Inserted in the AX5707 Maize Genome

{Refer to Figure 5 (Appendix 05. Kandel, Prem. RIR-0007265 - Volume 2 CBI, 2024)}

FIGURE 7. Alignment of the Genomic Insertion Site Sequence from Nontransgenic, near-isogenic AX5707 Maize to the 1000-bp 5' and 1000-bp 3' Genomic Sequences Flanking the MZIR260 Insert

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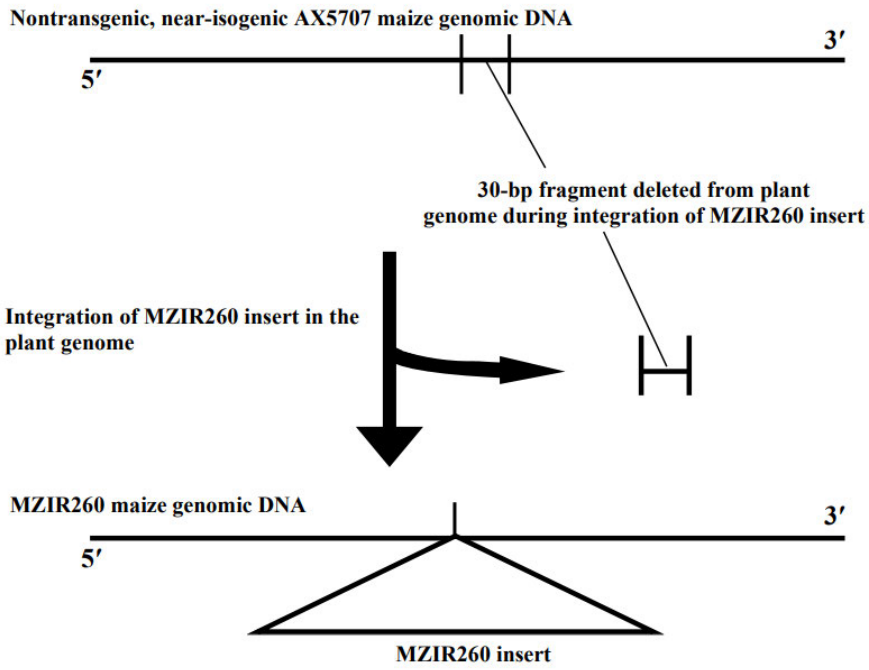

FIGURE 8. Representation of MZIR260 Genomic Insertion Site

c) Assessment to ascertain if the MZIR260 inserts disrupts endogenous maize genes

The study report to ascertain if the MZIR260 insert disrupts any endogenous maize genes is included as part of this submission dossier (Appendix 06).

Appendix 06. Kakeshpour, Tayebeh. RIR-0013777-23. (2024). *Event MZIR260 Maize: Basic Local Alignment Search Tool for Nucleotides (BLASTN) and Translated Nucleotides (BLASTX) Analyses of Maize Genomic Sequences Flanking the Insert. Final Report.* Unpublished. Syngenta Seeds, LLC.

In summary, the 5' and 3' genomic sequences flanking the MZIR260 insert in Event MZIR260 determined previously by Bartaula, R. 2024 (*unpublished*) – Appendix 02, were screened for similarity to deoxyribonucleic acid (DNA) sequences found in public databases, specifically the latest version of the National Center for Biotechnology Information (NCBI) nr/nt database (NCBI, 2023a), the NCBI Viridiplantae (taxid 33090) EST database (NCBI, 2023b), and protein sequences in NCBI non-redundant (nr) protein database (NCBI, 2023c). Sequence similarity analyses were performed with the BLASTN (version 2.8.1+) and BLASTX (version 2.8.1+) programs (Altshul *et al*., 1997).

BLASTN analyses using the nr/nt database and BLASTX analyses using the nr protein database resulted in no alignments for the maize genomic sequences flanking the MZIR260 insert.

BLASTN analyses using the Viridiplantae EST database resulted no alignments for the maize genomic sequence flanking the 3' region of the MZIR260 insert but did reveal one alignment

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for the maize genomic sequence flanking the 5' region of the MZIR260 insert (Table 3). The returned alignment was examined, and it did not provide evidence that a known maize gene was interrupted by the MZIR260 insert. The returned alignment from BLASTN analysis of the maize genome sequence flanking 5' region of the MZIR260 maize insert did show a short 61 bp region of homology to *Z. mays* with *E*-value of 1.30×10^{-14} located 216 bp upstream of the genome-to-insert junction. The alignment did not cover the flanking sequence immediately adjacent to the MZIR260 genome-to-insert junction and the 3' flanking sequence did not align to the same accession.

In Conclusion:

- BLASTX results combined with those from BLASTN analyses indicate the MZIR260 (T-DNA) insert does not disrupt any known endogenous maize gene.

TABLE 3 Results of BLASTN Analysis of 1000 bp of Maize Genomic Sequence Flanking the 5' Region of the MZIR260 Insert Using the Viridiplantae EST Database

Accession number	Description	*E*-value	Score (bits)
DW970250	DW970250 MSAM282720_3985_0264 LCM-dissected maize shoot apical meristem cDNA Zea mays cDNA, mRNA sequence.	1.30E-14	89.7

d) Determination of the chromosomal location of the transgenic locus

The study report to determine the chromosomal location of the MZIR260 transgenic locus by screening for similarity to the publicly available B73 maize reference genome sequence using BLASTN analysis, is included in this submission dossier (Appendix 07 and 08).

Appendix 07. Herrero, Sonia. RIR-0007258 - Volume 1_Amendment 1. (2023). *Event MZIR260 Maize. Determination of the Chromosomal Location of the Transgenic Locus. Assessment.* Unpublished, Syngenta Crop Protection, LLC.

Appendix 08. Herrero, Sonia. RIR-0007258 - Volume 2_Amendment 1. CBI. (2023). *Event MZIR260 Maize. Determination of the Chromosomal Location of the Transgenic Locus. Assessment.* Unpublished. Syngenta Crop Protection, LLC.

In summary, using the nucleotide sequence of the insertion site of Event MZIR260 in the maize genome as previously reported by Kandel, 2024 (*unpublished*) – Appendix 04, the chromosomal location of the MZIR260 transgenic locus was identified.

Using the BLASTN program, version 2.10.1+ (Zhang *et al*., 2000), the maize genomic sequence where MZIR260 insert integrated was screened for similarity to the deoxyribonucleic acid (DNA) sequences in maize using the latest version of the B73 maize reference genome (B73 RefGen_v5) (Woodhouse *et al*., 2021).

The current assembly for the representative B73 maize reference genome (i.e. B73 RefGen_v5) was used to perform BLASTN analyses. The genome assembly was constructed from PacBio®

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long reads and a mate-pair strategy. The resulting scaffolds were validated with BioNano optical mapping. The genome assembly was released in January 2020, and the database contained a total of 685 scaffold sequences, including 10 maize chromosomes (https://www.maizegdb.org/genome/assembly/Zm-B73-REFERENCE-NAM-5.0).

The BLASTN results described in Table 4 include the start and end base pair (bp) position of the chromosomal location of the BLAST hits, percent identity between MZIR260 genomic integration site and B73 maize, the *E-valu*e, and the score of alignments.

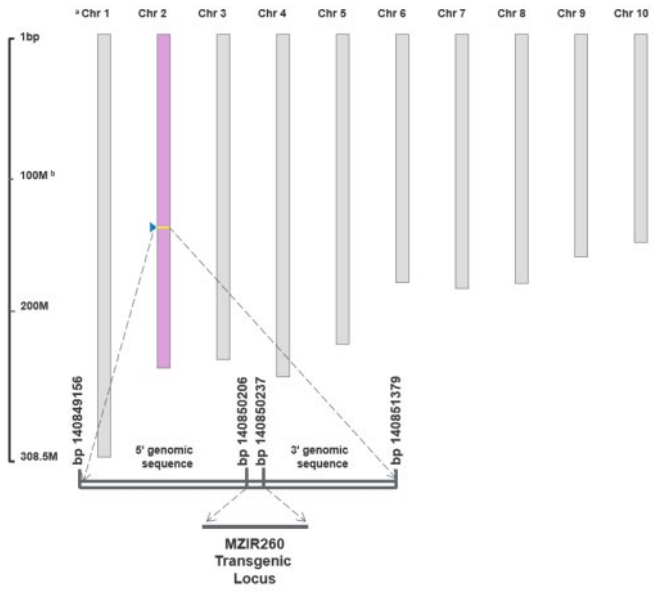
Several regions of maize chromosome 2 share homology to the genomic insertion site sequence of MZIR260. The maize region with highest similarity (99%) and with an *E*-value 0 is located in chromosome 2 between bp 140849156 and bp 140851379 (Figure 9).

In Conclusion:

- MZIR260 transgenic locus is located on maize chromosome 2.

TABLE 4 B73 Maize Chromosome Regions with Similarity to MZIR260 Genomic Insertion Site

Maize chromosome	Starting base pair	Ending base pair	Percent identity	*E*-value	Score
2	140849156	140851379	99	0	2221
2	141017217	141018496	88	0	809
2	141018952	141019701	89	0	509
2	141068524	141068636	97	3×10^{-46}	104
2	141304609	141304354	78	1×10^{-36}	87



[a] Chr = chromosome.
[b] M = mega bp.

FIGURE 9. Region of B73 Maize Chromosome 2 with Highest Similarity to MZIR260 Genomic Insertion Site

A schematic representation of the B73 maize chromosome 2 region with highest similarity to MZIR260 genomic insertion site and indicates the chromosomal location of the MZIR260 transgenic locus.

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(ii) A determination of the number of insertion sites, and the number of copies at each insertion site using whole genome sequencing

The study report detailing the use of whole genome sequencing to characterize the transgenic insert in MZIR260 maize including the generation used for creating commercial lines is included as part of this submission dossier (Appendix 09 and 10).

Appendix 09. Bartaula, Radhika. RIR-0007340 - Volume 1. (2024). *Event MZIR260 Maize: Insert Copy Number and Genetic Stability Analyses of T1, T3, and F1 Generations. Final Report.* Research Triangle Park, NC 27709-2257 USA: Unpublished. Syngenta Crop Protection, LLC.

Appendix 10. Bartaula, Radhika. RIR-0007340 - Volume 2. CBI. (2024). *Event MZIR260 Maize: Insert Copy Number and Genetic Stability Analyses of T1, T3, and F1 Generations. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

Whole Genome Sequencing (WGS) was performed to determine the presence or absence of transformation plasmid pSYN24795 backbone sequences in MZIR260 maize, the number of transferred deoxyribonucleic acid (T-DNA) integration sites (i.e., copy number) and to demonstrate the stability of the MZIR260 insert over three generations (T_1, T_3 and F_1) spanning five generations.

In summary, genomic deoxyribonucleic acid (gDNA) was extracted from MZIR260 T_1, T_3, and F_1 maize leaf tissue. The Illumina® NovaSeq™ 6000 Sequencing platform 150-bp paired-end (PE) mode at DNA Link Inc. was used to conduct WGS for all six samples (T_1, T_3, F_1, negative control, and two positive controls) and data was analyzed using an insertion site characterization (ISC) method outlined in Figure 9 (Cade *et al.*, 2018).

The test substance for this study is Event MZIR260 in the genetic backgrounds AX5707 maize for the T_1 and T_3 generations, and IJ7010 × AX5707 maize for the F_1 generation.

A DNA sequencing library was prepared at DNA Link, Inc. according to Illumina® TruSeq® DNA PCR-Free library preparation workflow. Six indexed genomic libraries were generated: three MZIR260 maize genomic libraries for each T_1, T_3, and F_1 generations; one for nontransgenic, near-isogenic negative control AX5707 maize; and two for positive assay controls spiked at one (1) copy and one-fourth (¼) copy per maize genome equivalent into AX5707 maize negative control DNA. The two positive controls demonstrated assay sensitivity.

The median sequence coverage depth of each sample sequenced was estimated from read alignments to the 10 single-copy BUSCO (Benchmarking Universal Single-Copy Orthologues) genes, representing one gene per maize chromosome. The mapping rate ranged from 99.25% to 99.96%, and median coverage across samples ranged from 168X to 193.5X.

a) Presence or absence of pSYN24795 plasmid backbone sequence

To determine the presence or absence of transformation plasmid pSYN24795 backbone sequences in MZIR260 maize, sequence read alignments of MZIR260 T_1, T_3, and F_1 sequences to pSYN24795 backbone region were evaluated using the Integrative Genomics Viewer (IGV).

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Reads aligning to the plasmid backbone sequences would indicate integration of backbone sequences in the maize genome during plant transformation.

Sequence reads for each of the three MZIR260 generations (T_1, T_3, and F_1) were aligned to pSYN24795 backbone region and no significant alignments were observed between the pSYN24795 backbone region and any of the reads from three MZIR260 maize generations.

The results from the analysis demonstrated that MZIR260 maize does not contain any pSYN24795 plasmid backbone sequences from the pSYN24795 transformation plasmid. However, for the MZIR260 maize T_1, T_3, and F_1 generations, 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 nontransgenic, near-isogenic AX5707 maize sample, thus indicating these reads were derived from other exogenous sources. Bacterial contaminants are common in greenhouse-procured tissue samples therefore this result is not unexpected (Zastrow-Hayes *et al*. 2015).

b) T-DNA insertion site(s) and insert copy number

Junction sequence analysis was conducted on regions spanning both the 5′ and 3′ genome-to-insert junctions to determine the number of integration sites and insert copy number. These junctions are unique and contain maize sequences flanking the MZIR260 insert and T-DNA sequences from the transformation plasmid pSYN24795 used to generate Event MZIR260.

Basically, the junction sequences were compared to pSYN24795, and for each generation (T_1, T_3 and F_1) two junctions were detected, one corresponding to the 5′ end and the other to the 3′ end of the MZIR260 inserted sequence, indicating the presence of a single MZIR260 insertion site.

In more detail, contigs that aligned to the pSYN24795 plasmid with > 97% identity, ≥ 25 bp length overlap, and ≤ 4% mismatches were selected and subsequently aligned to the AX5707 concatenated reference using Basic Local Alignment Search Tool for Nucleotides (BLASTN) to determine the genome-to-insert junction contigs and putative insertion site(s) in each of the MZIR260 maize generations (T_1, T_3, and F_1). Any contig that aligned entirely to T-DNA region of pSYN24795 without the presence of junction sequences were excluded from the analysis. Contigs containing genome-to-insert junction sequences were used to determine insert copy number and to further characterize the insertion site(s).

To determine the insert copy number, the number of unique 5′ end and 3′ end genome-to-insert junction sequences were determined and analysed. A summary and graphical representation of Event MZIR260 genome-to-insert junction sequence analysis of junction contigs and insertion site determination in MZIR260 T_1, T_3, and F_1 maize is shown in Figure 11.

Junction sequence analysis revealed that all three generations contain genome-to-insert junctions that are chimeric sequences consisting of maize DNA and MZIR260-specific sequence present in the T-DNA of the transformation plasmid pSYN24795. For each generation, two junction chimeric sequences, one on the 5′ end and another on the 3′ end of the insert, were identified, indicating the presence of a single insertion found between base pairs (bp) 144577192 and 144577223 on AX5707 maize chromosome 2. The sequence length, average and median per-base coverage obtained for both 5' and 3' junctions for each generation are shown in Table 5.

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Note, using the B73 maize reference genome as previously reported by Herrero, S. (*unpublished*) 2023 – Appendix 07, the location was mapped to chromosome 2, between bp 140849156 and 140851379. This was due to using a different maize reference sequence.

c) Gene stability

A single integration at the same locus and 100% identity of the genome-to-insert junctions for all three generation of MZIR260 maize (T_1, T_3, and F_1) demonstrated the stable inheritance of the MZIR260 insert spanning five generations and established genetic identity across the MZIR260 maize pedigree.

In Conclusion, WGS analysis demonstrated:

- The MZIR260 maize does not contain pSYN24795 plasmid backbone sequences from the pSYN24795 transformation plasmid
- The insert is present as a single copy in chromosome 2 in MZIR260 maize
- Stable inheritance of the MZIR260 insert over multiple generations

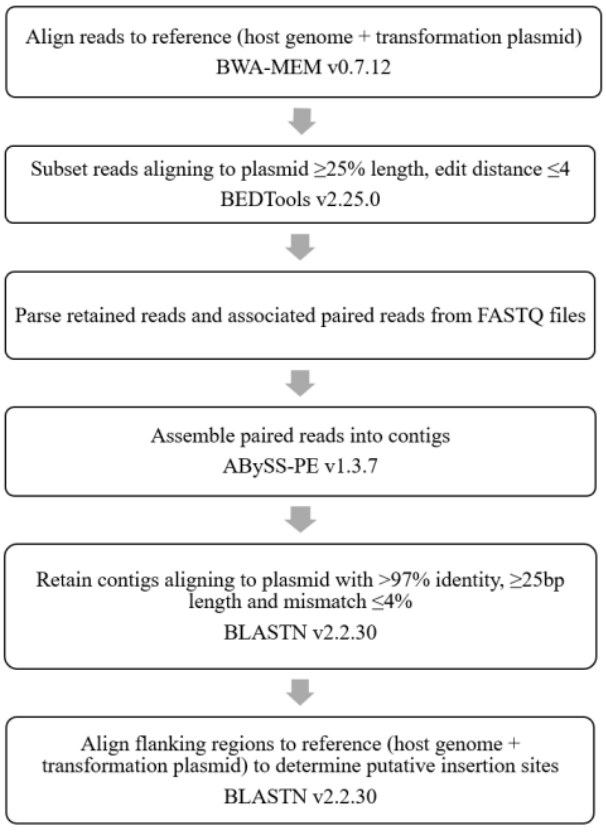

FIGURE 10. Flowchart of the Bioinformatic Analysis Using the ISC Method

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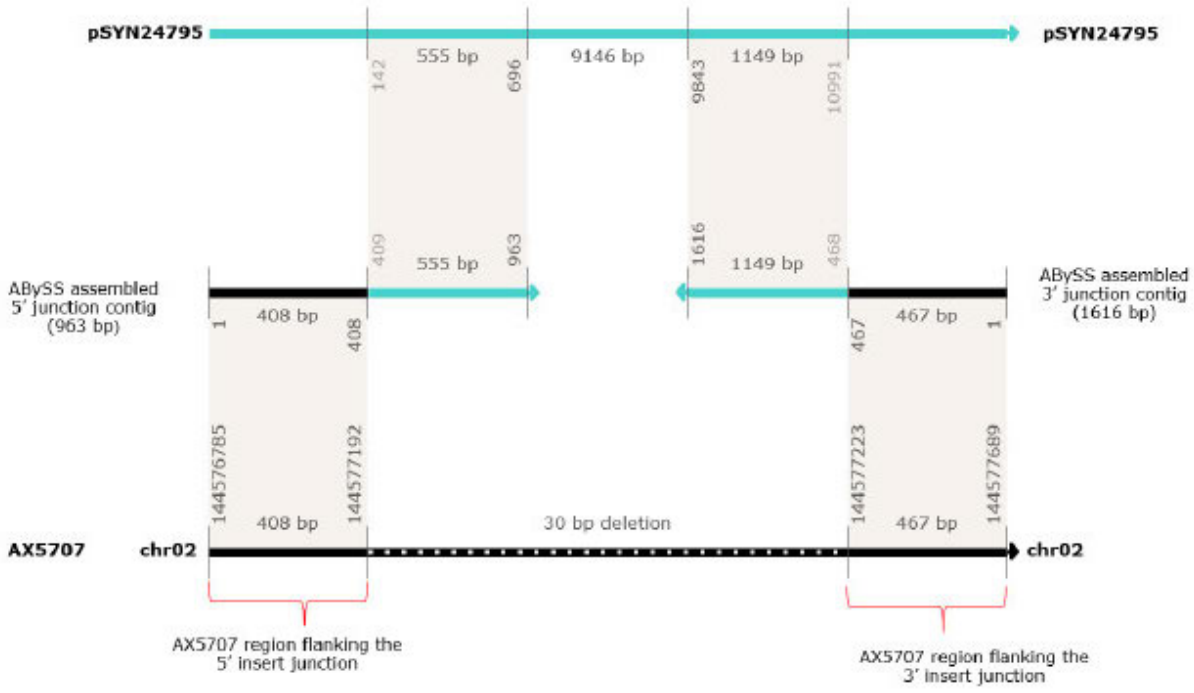

Figure 11 (a). Junction Sequence Analyses and Insertion Site of Event MZIR260 (T_1 Maize)

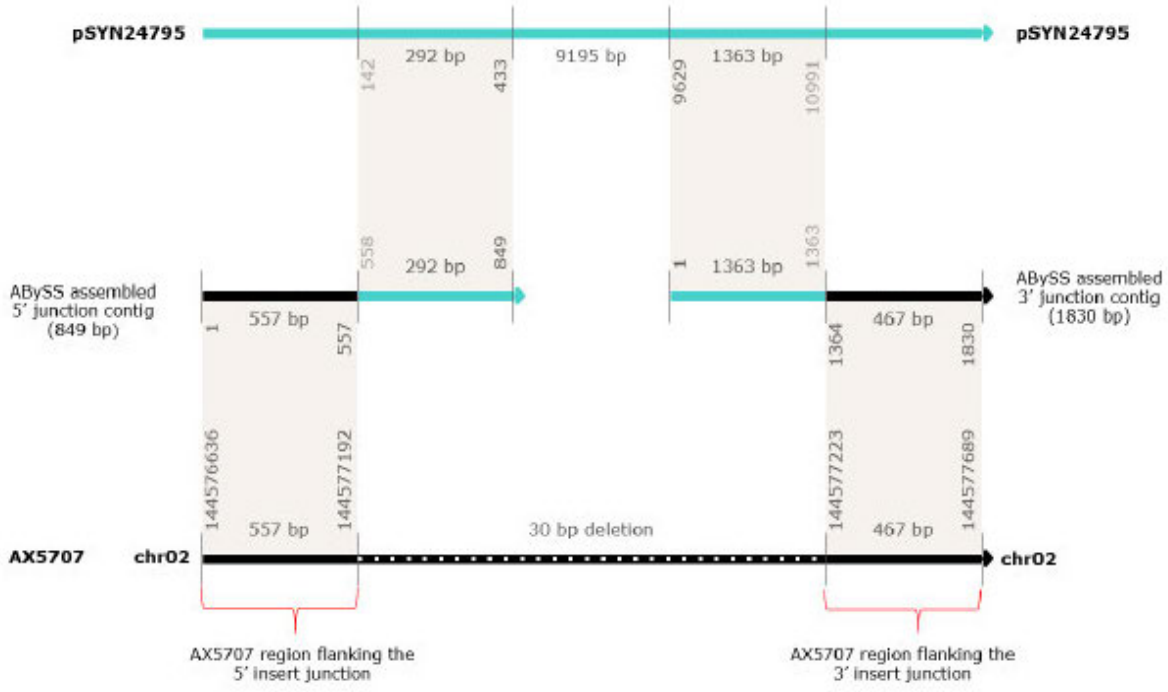

Figure 11 (b). Junction Sequence Analyses and Insertion Site of Event MZIR260 (T_3 Maize)

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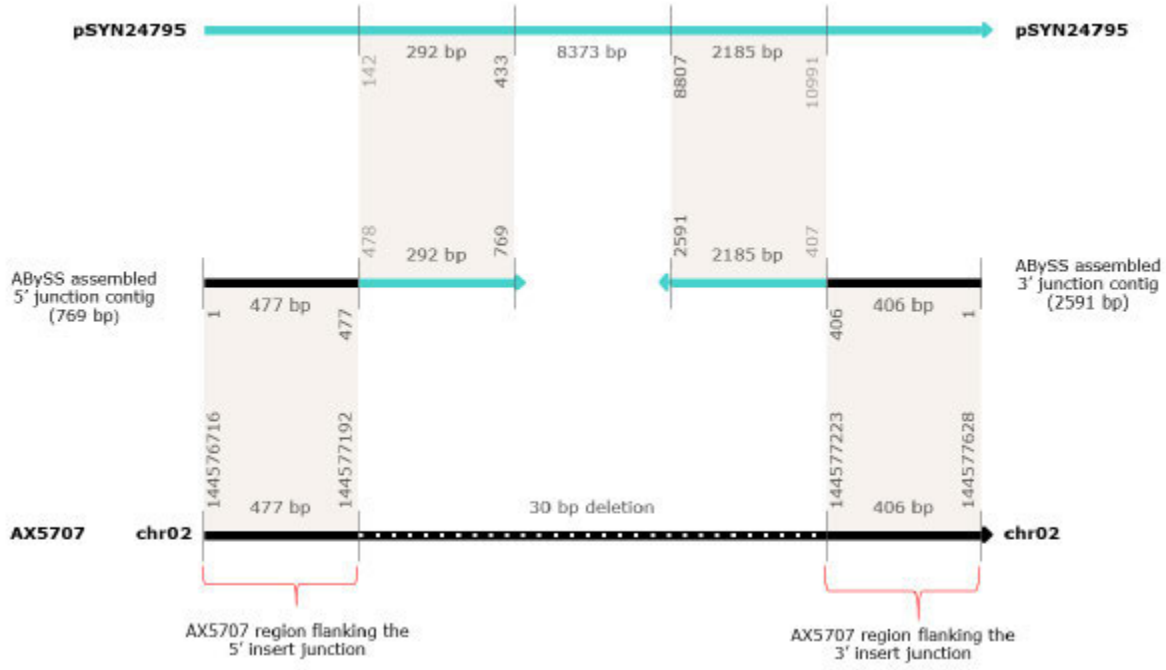

Figure 11 (c). Junction Sequence Analyses and Insertion Site of Event MZIR260 (F_1 Maize)

FIGURE 11. Junction Sequence Analyses and Insertion Site of Event MZIR260 over Three (3) Generations

Notes for Figures 11 (a, b, c): Sequences specific to the transformation plasmid pSYN24795 and to the nontransgenic, near-isogenic AX5707 maize genome are depicted by turquoise and black arrows, respectively. The **top line** (turquoise) represents the pSYN24795 reference sequence displayed as a linear molecule (16208 bp); the **middle lines** represent genome-to-insert junction contig sequences derived from MZIR260 maize and composed of AX5707 maize-specific (black) and pSYN24795 T-DNA-specific (turquoise) sequences; and the **bottom line** (black) represents AX5707 maize sequence. For junction contigs (**middle lines**), regions of homology to either the pSYN24795 reference sequence (**top line**), or the 5' and 3' regions from AX5707 maize sequences flanking the MZIR260 insert (**bottom line**) are shaded in beige. The direction of the arrows represents forward strand (→) or reverse complementary strand (←). For each region of homology among these sequences (**top, middle, bottom**), the location coordinates and the size in bp are given. The patterned portion in AX5707 maize genome (**bottom line**) represents a 30-bp deletion at the insertion site where Event MZIR260 integrated in chromosome 2 (chr02). Diagrams are representations of sequence homology and not scaled to the bp length

TABLE 5. Length and Coverage of Junction Sequences

Sample	Sequence length of junction contig (bp)		Median coverage of junction contig		Average coverage of junction contig	
	5'	3'	5'	3'	5'	3'
MZIR260 T_1	963	1616	98 X	74 X	83 X	67 X
MZIR260 T_3	849	1830	86 X	164 X	95 X	139 X
MZIR260 F_1	769	2591	51 X	103 X	54 X	100 X

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(iii) Map(s) illustrating the organisation of the inserted genetic material in planta

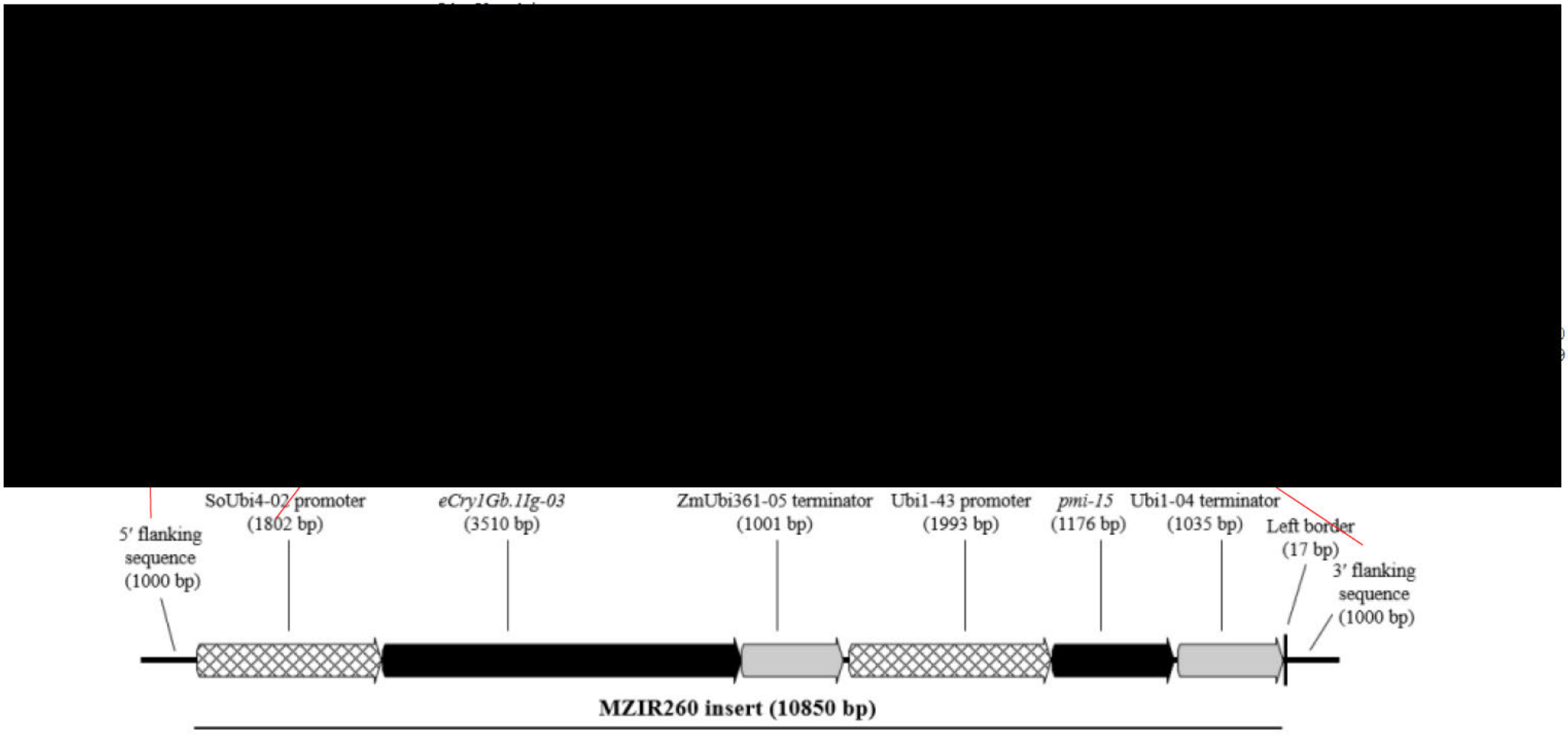

FIGURE 12. Organisation of the inserted genetic materials *in planta:* T-DNA from pSYN24795 transferred to MZIR260

(iv) Analysis for occurrence of open reading frames (ORFs) in insert and junction regions

Hypothetical ORF sequences were investigated for biologically relevant sequence similarity to known or putative protein allergens and/or toxins that might represent a risk of allergic cross-reactivity or toxicity to human or animal consumers of MZIR260 maize or its by-products.

Appendix 11. Joshi, Saurabh. RIR-0006836-23. (2024). *Event MZIR260 Allergenicity and Toxicity Assessment of Start to Stop, Genome to Insert Junction Open Reading Frames. Assessment.* Unpublished. Syngenta Seeds, LLC.

Appendix 12. Joshi, Saurabh. RIR-0006835-23. (2024). *Event MZIR260 Allergenicity and Toxicity Assessment of Start-to-Stop Open Reading Frames within the MZIR260 Insert. Assessment.* Unpublished. Syngenta Seeds, LLC.

Bioinformatic analyses were used to evaluate *in silico* translations of short deoxyribonucleic acid (DNA) sequences that hypothetically encode open reading frames (ORFs) of 30 or more amino acids with translations on each of the six possible coding frames of the DNA between a start codon (ATG) and a stop codon (TAA, TAG, TGA) that span the junctions between the maize genomic sequence and the MZIR260 insert (Appendix 11). The purpose of these analyses was to determine if any of the amino acid translations of the hypothetical junction ORFs have sequence similarity to known or putative protein allergens and/or toxins that might represent a risk of allergenicity or toxicity to human or animal consumers of MZIR260 maize or its by-products.

Bioinformatic analyses were used to evaluate *in silico* translations of short deoxyribonucleic acid (DNA) sequences that hypothetically encode open reading frames (ORFs) of 30 or more amino acids (aa) with translations on each of the six possible coding frames of the DNA between a start codon (ATG) and any of the stop codons (TAA, TAG, TGA) that span the MZIR260 insert (Appendix 12). The purpose of these analyses was to determine if any of the amino acid translations of the hypothetical insert ORFs have sequence similarity to known or putative protein allergens and/or toxins that might represent a risk of allergenicity or toxicity to human or animal consumers of MZIR260 maize or its by-products.

The nucleotide sequence used in both analyses, either the region that spans the MZIR260 insert DNA or the junctions of the maize genome and the insert DNA region, was identical to that reported by Bartaula, R. 2024 (*unpublished*) – Appendix 02.

For assessments of allergenicity, two alignment searches were performed. First, a FASTA, (FAST-All sequence alignment software package) full-length sequence search was used to identify alignments for each of the translated insert ORFs with the sequences in the Comprehensive Protein Allergen Resource (COMPARE) allergen database (version 2023). For translated insert ORFs of 80 or more amino acid, the alignments were evaluated with a minimum criterion of greater than 35.0% shared amino acid identity over an alignment overlap length of at least 80 amino acids. For translated insert ORFs with less than 80 ($\geq$ 30) amino acid, the expectation value (*E*-value) of 1×10^{-6} or lower was used to identify potentially relevant alignments. Second, all translated insert ORFs were analyzed for exact matches of contiguous 8-amino acid sequences (8mer) between the query and all allergen sequences in the COMPARE allergen (version 2023) database. Any identified exact match of continuous 8mers would indicate a need for further evaluation.

For assessments of toxicity, each ORF translation was compared using the Basic Local Alignment Search Tool for Proteins (BLASTP) algorithm (version 2.8.1+) to amino acid sequences in a 2023 curated database based on the publicly available set of relevant toxin protein sequences from the UniProt database to determine the extent of its potential sequence similarity to known or putative toxins. Any alignments with *E*-values less than 1×10^{5} were evaluated further.

a) Start-to-stop ORFs spanning the MZIR260 Insert - Junction Region

No translated hypothetical ORF sequences were identified at the junctions between the maize genomic sequence and the MZIR260 insert. Therefore, no bioinformatic comparisons were conducted with the allergen or toxin database. This concludes that no amino acid translations of the hypothetical junction ORFs share biologically relevant sequence similarity to known or putative protein allergens or toxins of mammalian concern (Appendix 11).

b) Start-to-stop ORFs spanning the MZIR260 Insert Region

Thirty – five hypothetical ORF sequences were identified that span the MZIR260 insert as presented in Table 6 (Appendix 12). Of the 35 ORFs, 5 ORFs were more than 80 amino acids while 30 ORFs were less than 80 (≥ 30) amino acids. No hypothetical amino acid translations share biologically relevant sequence similarity to known or putative protein allergens or toxins of mammalian concern.

For the FASTA, full-length sequence allergen search, there was no significant similarity observed between the hypothetical insert ORF sequences and any entry in the COMPARE database (version 2023).

The 8mer exact match search identified an expected match between a MZIR260-derived sequence and a frog allergen, which has previously been demonstrated to have no allergenic cross-reactivity. Thus, the results revealed ***no biologically significant*** sequence similarity between any of the translated insert sequences and any entry in the COMPARE database (version 2023).

For assessments of toxicity, no significant sequence similarity (*E-value* $< 1\times10^{-5}$) was observed between any of the translated hypothetical ORF sequences and any entry in the 2023 curated database based on the publicly available set of relevant toxin protein sequences from the UniProt database.

In Conclusion:

- No hypothetical amino acid translations share biologically relevant sequence similarity to known or putative protein allergens or toxins of mammalian concern.

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TABLE 6. Open Reading Frames Derived from MZIR260 Insert (30 or more amino acids)

No.	ORFs	Length	Sequence
1	MZIR260_insert_1 [682 - 798]	39	MACSRPSICSRFAGRGVDLRAVMKLFGVICSPDSAGWLE
2	MZIR260_insert_2 [804 - 947]	48	MMVGPVGSFTALGLGWDDVACAVARDPAAGLAFDCQISLRLCDLVWTF
3	MZIR260_insert_3 [853 - 1026]	58	MMLHAPLRVIPQQDLRLIARSRYDYVIWFGLFRSVASAYVPDAPTAHMPDDNHKWLWN
4	MZIR260_insert_4 [1787 - 1933]	49	MYQFFCVQQSVFVRFIVTYGRLLFWSSMLAGILNHGDQQPEPVRAVQLP
5	**MZIR260_insert_5 [1890 - 5396] (eCry1Gb.1g)**	**1169**	**MEINNQNQCVPYNCLNNPESEILNVAIFSSEQVAEIHLKITRLILENFLPGGSFAFGLFD LIWGIFNEDQWSAFLRQVEELINQRITEFARGQAIQRLVGFGRSYDEYILALKEWEND PDNPASKERVRTRFRTTDDALLTGVPLMAIPGFELATLSVYAQSANLHLALLRDAVFF GERWGLTQTNINDLYSRLKNSIRDYTNHCVRFYNIGLGNLNVIRPEYYRFQRELTISV LDLVALFPNYDIRTYPIPTKSQLTREIYTDPIISPGAQAGYTLQDVLREPHLMDFLNRLI IYTGEYRGIRHWAGHEVESSRTGMMTNIRFPLYGTAATAEPTRFITPSTFPGLNLFYR TLSAPIFRDEPGANIIIRYRTSLVEGVGFIQPNNGEQLYRVRGTLDSLDQLPLEGESSLT EYSHRLCHVRFAQSLRNAEPLDYARVPMFSWTHRSATPTNTIDPDVITQIPLVKAHTL QSGTTVVKGPGFTGGDILRRTSGGPFAFSNVNLDWNLSQRYRARIRYASTTNLRMYV TIAGERIFAGQFNKTMNTGDPLTFQSFSYATIDTAFTFPTKASSLTVGADTFSSGNEVY VDRFELIPVTATFEAEYDLEKAQKAVNALFTSSNQIGLKTDVTDYHIDKVSNLVECLS DEFCLDEKRELSEKVKHAKRLCDERNLLQDPNFRGINRQPDRGWRGSTDITIQGGDD VFKENYVTLPGTFDECYPTYLYQKIDESKLKAYTRYELRGYIEDSQDLEIYLIRYNAK HETVNVPGTGSLWPLSAQSPIGKCGEPNRCATHLEWNPDLDCSCRDGEKCAHHSHH FSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEK KWRDKREKLELETNIVYKEAKKSVDALFVNSQYDRLQADTNIAIIHAADKRVHSIREA YLPELSVIPGVNAAIFEELEGRIFTAYSLYDARNVIKNGDFNNGLSCWNVKGHVDVEE QNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIEDNTDEL KFSNCVEEEIYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSVPADYASAYE EKAYTDGRRDNTCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETEGTFIVDS VELLLMEE**
6	MZIR260_insert_6 [5597 - 5764]	56	MLFEFKLFAEYCCFLSCQLMLLNWLPSNFCLLVFVAPYLFTVCLLQVLAVSFFVTS
7	MZIR260_insert_7 [7699 - 7812]	38	MIFFVSLHRVWFALFLYFNICRALVCRVIFSCFFLSWL
8	MZIR260_insert_8 [7758 - 7886]	43	MPCTCLSGHLFMLFFVLVVMMWSGWAVVLDRSRILFQTTWWIY

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TABLE 6. Continued

9	**MZIR260_insert_9 [8468 - 9640] (PMI)**	**391**	**MQKLINSVQNYAWGSKTALTELYGMENPSSQPMAELWMGAHPKSSSRVQNAAGDIV SLRDVIESDKSTLLGEAVAKRFGELPFLFKVLCAAQPLSIQVHPNKHNSEIGFAKENAA GIPMDAAERNYKDPNHKPELVFALTPFLAMNAFREFSEIVSLLQPVAGAHPAIAHFLQ QPDAERLSELFASLLNMQGEEKSRALAILKSALDSQQGEPWQTIRLISEFYPEDSGLFS PLLLNVVKLNPGEAMFLFAETPHAYLQGVALEVMANSDNVLRAGLTPKYIDIPELVA NVKFEAKPANQLLTQPVKQGAELDFPIPVDDFAFSLHDLSDKETTISQQSAAILFCVEG DATLWKGSQQLQLKPGESAFIAANESPVTVKGHGRLARVYNKL**
10	MZIR260_insert_10 [9705 - 9851]	49	MCLRRLVPSLHMEVVKVSAVRVMSRVSVGLIMDRLCCVCVLPRTMTNHE
11	MZIR260_insert_11 [9860 - 9964]	35	MFEIKACAHYVLSFSCLLIFASRYWLSTVSYLGGV
12	MZIR260_insert_12 [10414 - 10560]	49	MHDSNLITPAEVDTVTRFLNLFTKSLENTHTHSCQSWLEKFHDKMPKLS
13	MZIR260_insert_13 [10538 - 10645]	36	MTKCQSCLDSSLLAMSRDLVWFNGPVLLACSTQNCC
14	MZIR260_insert_14 [9492 - 9238] (REVERSE SENSE)	85	MAALCWLMVVSLSLRSCSEKAKSSTGIGKSSSAPCFTGWVNNWLAGLASNFTLATSSGIS MYLGVRPARSTLSEFAITSSATPCR
15	MZIR260_insert_15 [8754 - 8536] (REVERSE SENSE)	73	MESGCAAHNTLNRKGSSPKRLATASPSRVDLSLSITSRSETISPAAFCTRELLFGCAPIHSSAI GWLDGFSIP
16	MZIR260_insert_16 [8590 - 8489] (REVERSE SENSE)	34	MCAHPQLGHRLAGRIFHTVKFSQRRFAAPGIVLH
17	MZIR260_insert_17 [8481 - 8392] (REVERSE SENSE)	30	MSFCMIGDPCRSNTKQQGEQRQKKQYQANK
18	MZIR260_insert_18 [8258 - 8166] (REVERSE SENSE)	31	MNRCCICHHVYASVKPTSTCIPILDRYFHPS
19	MZIR260_insert_19 [8250 - 8158] (REVERSE SENSE)	31	MLHMPSCICISKTHINMYTYPRSIFPSILNS
20	MZIR260_insert_20 [8064 - 7930] (REVERSE SENSE)	45	MNDRPTTPHHHNQANKKHLCICISKTRINMYTYPRSIFPSIIFNS
21	MZIR260_insert_21 [7722 - 7582] (REVERSE SENSE)	47	MQRNKKNHEIDPVCGTARAIPGFPKEKHWQVSNQNVSDVQVASVYER
22	MZIR260_insert_22 [7700 - 7551] (REVERSE SENSE)	50	MKSIPSAERLEPSQDSPKRNTGKLAIRTCLTYRSHPCTNANSTDLTQTRI
23	MZIR260_insert_23 [7508 - 7071] (REVERSE SENSE)	146	MDRNADLEKVERGGGGGRAAYLEAEVPTGGFGGDLVVCVCAPNNTRLGKEGVEGVSIY YGGRGRESEGAVGKESPVAAGAVRGGGGRLPCRLTSAAPPRNFWMPTAEQVQRWSGTLE RGPEAATEMPCRLLRLARRDAAGSLVGVR
24	MZIR260_insert_24 [6817 - 6725] (REVERSE SENSE)	31	MDVLIKWMKYYIGEAICKKKGEHMHTKKIKL

(d) A family tree and/or description of the breeding process including which generation have been used for each study

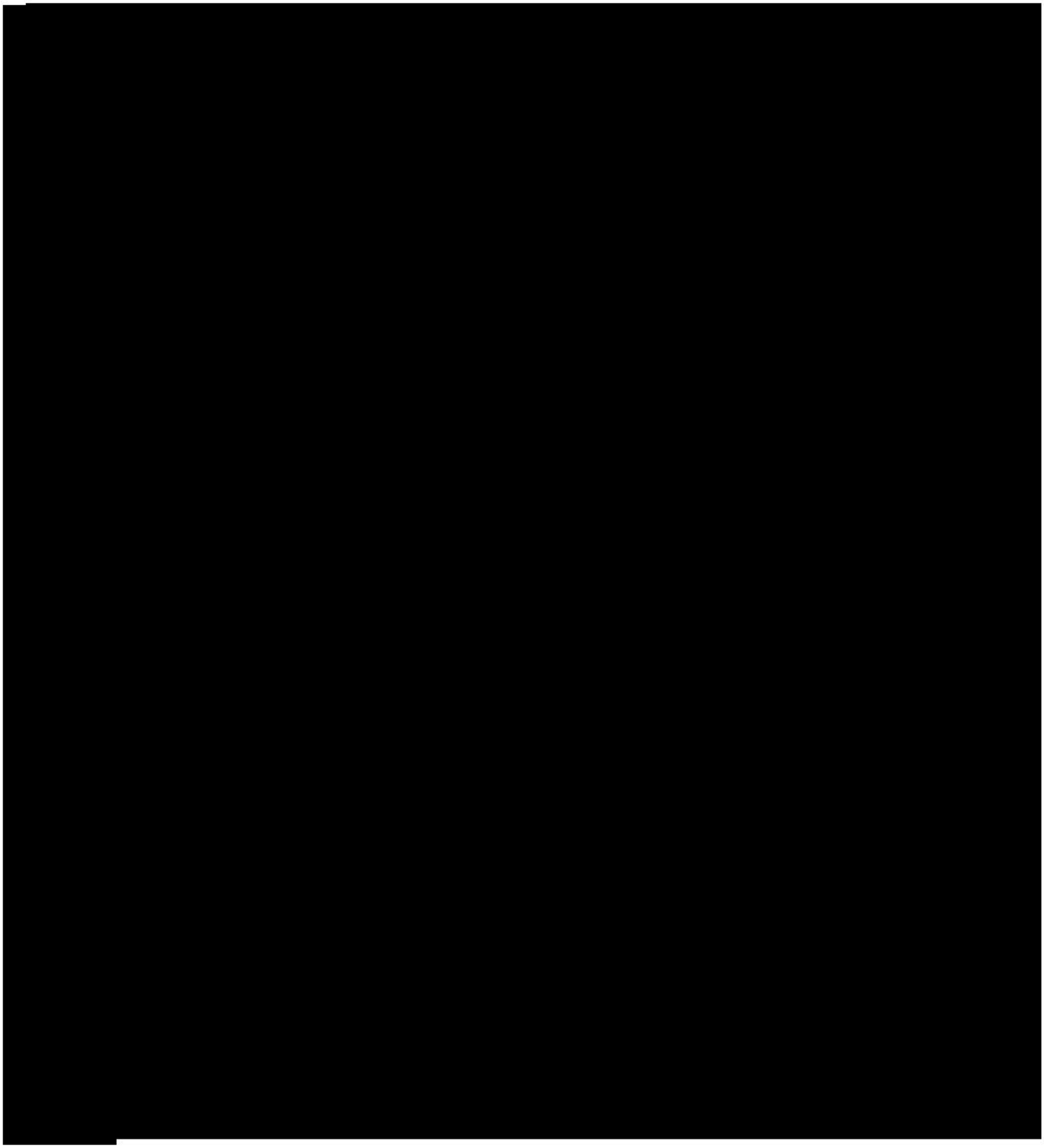

TABLE 7. Pedigree /Generation Used in Each Study

Generation	Material ID	Study	RIR
MZIR260 T_3 Maize	[redacted]	Insert and Flanking Sequence	[redacted]
MZIR260 T_3 Maize	[redacted]	Genomic Insertion Site Analysis	[redacted]
MZIR260 T_1 Maize MZIR260 T_3 Maize MZIR260 F_1 Maize	[redacted]	Insert Copy Number and Genetic Stability Analyses of T_1, T_3, and F_1 Generations WGS	[redacted]
MZIR260 F_1 Maize	[redacted]	Quantification of eCry1Gb.1Ig, and Phosphomannose Isomerase in Event MZIR260 Maize Tissues	[redacted]
MZIR260 F_1 Maize	[redacted]	Compositional Analysis of Forage and Grain from Event MZIR260 Maize Grown in the USA in 2022	[redacted]
MZIR260 F_1 maize	[redacted]	Comparison of eCry1Gb.1Ig Protein Expressed in the Event MZIR260 Derived Maize Plants and eCry1Gb.1Ig Protein Expressed in Recombinant *Escherichia Coli*	[redacted] 9521, 2024)
MZIR260 F_1 maize	[redacted]	Comparison of Phosphomannose Isomerase (PMI) Protein Expressed in the Event MZIR260 Derived Maize Plants and PMI Protein Expressed in Recombinant *Escherichia Coli*	[redacted]
MZIR260 BC_2F_1 Maize MZIR260 BC_3F_1 Maize MZIR260 F_2 Maize	[redacted]	Mendelian Inheritance	[redacted]
MZIR260 BC_2F_1 Maize MZIR260 BC_4F_1 Maize MZIR260 BC_5F_1 Maize	[redacted]	Quantification of eCry1Gb.1Ig and Phosphomannose Isomerase in Tissues from Multiple Generations of Maize Derived from Transformation Event MZIR260	[redacted]

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(e) Stability of the genetic changes

(i) WGS to demonstrate gene stability in T_1, T_3 and F_1 MZIR260 maize generations

Evidence of the stability of the genetic modification at the genotypic level is demonstrated in Section 2.03 c (ii) on page 37, by Radhika, B (*unpublished*) 2024 – Appendix 09.

In summary, WGS analysis of MZIR260 T_1, T_3, and F_1 maize demonstrated identical sequences for the 5′ genome-to-insert junctions and the 3′ insert-to-genome junctions from all three generations and a single integration site in the Event MZIR260 maize genome. The results also confirmed a single integration at the same locus and 100 % identity of the genome-to-insert junctions for all three generations of MZIR260 maize demonstrating the stable inheritance of the MZIR260 insert over five generations and established genetic identity across the MZIR260 maize pedigree.

(ii) Mendelian inheritance to demonstrate pattern of inheritance in three segregating generations of Maize - BC_2F_1, BC_3F_1, and F_2

A study confirming Mendelian inheritance using real-time quantitative polymerase chain reaction (qPCR) to determine segregation ratios and Chi-square analysis of the segregation data is included as part of this application dossier (Appendix 13 and 14).

Appendix 13. Lee, Tae-Jin. RIR-0007260 - Volume 1. (2023). *Event MZIR260 Maize Mendelian Inheritance Analysis. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

Appendix 14. Lee, Tae-Jin. RIR-0007260 - Volume 2 CBI. (2023). *Event MZIR260 Maize Mendelian Inheritance Analysis. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

In summary, Mendelian inheritance of the trait gene, *eCry1Gb.1Ig-03*, and the selectable marker gene, *pmi-15*, present in Event MZIR260 was assessed in three segregating generations (BC_2F_1, BC_3F_1, and F_2) of MZIR260 maize. Backcross (BC) generations for MZIR260 were produced by crossing a hemizygous MZIR260 maize parent (F_1) to a non-transgenic recurrent parent (BDAX4608), and this backcrossing was repeated through several breeding cycles to yield the BC_2F_1 and BC_3F_1 generations. In addition, the F_1 generation was selfed to yield the F_2 generation. A total of 210, 218, and 132 plants were individually analysed from BC_2F_1, BC_3F_1, and F_2 generations, respectively, to determine copy number of *eCry1Gb.1Ig-03* and *pmi-15* by real-time quantitative PCR (qPCR) analysis (Ingham *et al.* 2001) using *eCry1Gb.1Ig-03*-specific and *pmi-15*-specific primers and probes. The copy number for *eCry1Gb.1Ig-03* and *pmi-15* determined by real-time qPCR from individual plants in the BC_2F_1 and BC_3F_1, and F_2 generations was used to calculate gene frequency and observed segregation ratios.

Real-time qPCR analysis confirmed the copy number of eCry1Gb.1Ig-*03* and *pmi-15* in a portion of the plants in all three generations. The genes *eCry1Gb.1Ig-03* and *pmi-15* co-segregated (i.e.,

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when one gene was present, the other gene was also present). In the control assay, all plants tested positive for *adh1*, indicating that maize DNA was present in all reactions.

The frequencies for *eCry1Gb.1Ig-03* and *pmi-15* were identical in the three generations, and the two genes segregate as a single locus. The expected segregation ratio for *eCry1Gb.1Ig-03* and *pmi-15* in the BC_2F_1 and BC_3F_1 generations is 1:1 (i.e., 50% of the plants in each generation contain one copy of the transgenes and 50% of the plants in each generation do not contain the transgenes). The expected segregation ratio for *eCry1Gb.1Ig-03* and *pmi-15* in the F_2 generation is 1:2:1 (i.e., 25% of the plants contain two copies of the transgenes, 50% of the plants contain one copy of the transgenes, and 25% of the plants do not contain the transgenes).

The goodness-of-fit of the observed segregation ratios to the expected segregation ratios was tested by chi-square analysis:

$$\chi 2 = \text{sum (observed - expected)}^2 / \text{expected}$$

The chi-square critical value at $\alpha = 0.05$ is 3.84 for the BC_2F_1 and BC_3F_1 generations and 5.99 for the F_2 generation (Strickberger MW, 1976). The chi-square value is less than 3.84 for the BC_2F_1 (0.933) and BC_3F_1 (1.174) generations and less than 5.99 for the F_2 (2.970) generation tested indicating that *eCry1Gb.1Ig-03* and *pmi-15* in MZIR260 maize are inherited in a predictable manner, according to Mendelian principles. The gene frequencies and the results of the chi-square analysis in the BC_2F1, BC_3F1, and F_2 generations are shown in Table 8. Chi-square *critical value* at $\alpha = 0.05$ is 3.84 for the BC_2F_1 and BC_3F_1 generations and 5.99 for the F_2 generation.

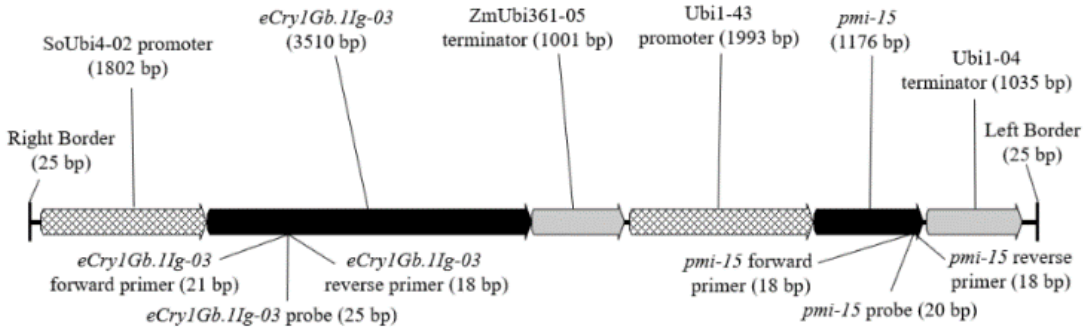

FIGURE 14. Locations of the Real-time qPCR Primers and Probes in the pSYN24795 Plasmid T-DNA

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TABLE 8. Observed and Expected Frequencies of *eCry1Gb.1Ig-03* and *pmi-15* in the BC2F1, BC3F1, and F2 Generations of MZIR260 Maize

eCry1Gb.1Ig-03* and *pmi-15	**BC_2F_1**		**BC_3F_1**		**F_2**	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Positive (2 copies)[a]	0	0	0	0	26	33
Positive (1 copy)[a]	98	105	117	109	66	66
Negative (null)[a]	112	105	101	109	40	33
Total[b]	210	210	218	218	132	132
χ^2	0.933[c]		1.174[c]		2.970[d]	

[a]Positive or negative for both *eCry1Gb.1Ig-03* and *pmi-15*
[b]Number of plants assessed
[c]*Probability* < 0.05 (χ^2 < 3.84)
[d]*Probability* < 0.05 (χ^2 < 5.99)

.

In Conclusion:

- The MZIR260 insert is inherited according to Mendelian principles and integrated into a chromosome within the nuclear genome of MZIR260 maize.

(iii) ELISA to demonstrate phenotype stability in BC_2F_1, BC_4F_1 and BC_5F_1 MZIR260 maize generations

A study detailing the use of enzyme-linked immunosorbent assay (ELISA) to measure the concentrations of eCry1Gb.1Ig and PMI in leaves, roots, pollen, forage, and kernels from maize plants of three generations derived from transformation Event MZIR260 is provided as part of this application dossier (Appendix 15).

Appendix 15. Read, Alysha. RIR-0010739. (2024). *Quantification of eCry1Gb.1Ig and Phosphomannose isomerase in Tissues from Multiple Generations of Maize Derived from Transformation Event MZIR260 Final Report.* Unpublished. Syngenta Crop Protection, LLC.

In summary, MZIR260 maize plants of three generations (BC_2F_1, BC_4F_1 and BC_5F_1) were grown according to standard greenhouse practices. Tissue samples were collected from plants at the vegetative stage six (V6), reproductive stage one (R1), reproductive stage four (R4), and reproductive stage six (R6). Samples of each tissue type were collected from five replicate plants per generation. ELISA was used to quantify eCry1Gb.1Ig and PMI in each MZIR260 maize tissue sample. Analysis of nontransgenic, near-isogenic maize tissue extracts confirmed the absence of plant-matrix effects on the ELISA methods.

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From each plot, leaves and roots were collected from maize plants at three different growth stages, pollen and forage were collected at one growth stage, and kernels were collected from maize plants at two growth stages.

- Table 9 shows the concentrations of eCry1Gb.1Ig protein in R6 and R6 senescence kernels and from R4 forage from MZIR260 maize plants of multiple generations on a DW and a FW basis.
- Table 10 shows the concentrations of PMI protein in R6 and R6 senescence kernels and from R4 forage from MZIR260 maize plants of multiple generations on a DW and a FW basis.
- Table 11 shows the concentrations of eCry1Gb.1Ig and PMI protein in Leaves from MZIR260 maize plants of multiple generations on a DW and a FW basis.
- Table 12 shows the concentrations of eCry1Gb.1Ig and PMI protein in Roots from MZIR260 maize plants of multiple generations on a DW and a FW basis.
- Table 13 shows the concentrations of eCry1Gb.1Ig and PMI protein in Pollen from MZIR260 maize plants of multiple generations on a DW and a FW basis.

In conclusion:

- Concentrations of eCry1Gb.1Ig and PMI measured in MZIR260 maize in V6, R1, R6 leaves and roots, R1 pollen, R4 forage, and R6 and R6 senescence kernels were similar across all three generations.

- eCry1Gb.1Ig and PMI protein expression in MZIR260 maize is consistent from generation to generation.

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TABLE 9. Concentrations of eCry1Gb.1Ig Protein in R6 and R6 Senescence Kernels and from R4 forage from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis

Generation	Kernels (R6)				Kernels (R6 senescence)				Forage (R4)			
	µg/g DW		µg/g FW		µg/g DW		µg/g FW		µg/g DW		µg/g FW	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
BC_2F_1	238.33	102.70 – 495.48	197.60	84.96 – 399.88	126.66	91.31 – 167.26	117.08	83.00 – 155.49	354.64	191.02 – 434.31	113.04	68.01 – 131.05
BC_4F_1	141.35	96.01 – 197.08	116.43	78.54 – 158.31	111.69	89.32 – 142.40	102.91	81.89 – 131.48	354.76	214.63 – 485.28	115.74	79.30 – 155.22
BC_5F_1	112.00	61.00 – 140.85	96.21	52.09 – 120.26	128.19	81.66 – 171.35	118.09	75.06 – 157.96	309.11	192.69 – 462.78	102.81	66.43 – 153.4

Table 9 shows on a dry weight (DW) basis, the range of eCry1Gb.1Ig protein concentrations in MZIR260 maize were 61.00 to 495.48 µg/g DW in R6 kernels, 81.66 to 171.35 µg/g DW in R6 senescence kernels and 191.02 to 485.28 µg/g DW in R4 forage. On a fresh weight (FW) basis, the range of eCry1Gb.1Ig protein concentrations in MZIR260 maize were 52.09 to 399.88 µg/g FW in R6 kernels, 75.06 to 157.96 µg/g FW in R6 senescence kernels and 66.43 to 155.22 µg/g FW in R4 forage.

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TABLE 10. Concentrations of PMI Protein in R6 and R6 Senescence Kernels and from R4 forage from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis

Generation	Kernels (R6)				Kernels (R6 senescence)				Forage (R4)			
	µg/g DW		µg/g FW		µg/g DW		µg/g FW		µg/g DW		µg/g FW	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
BC2F1	2.65	2.60 – 2.74	2.21	2.13 – 2.40	2.70	2.54 – 2.82	2.49	2.36 – 2.60	14.54	11.70 – 15.68	4.75	3.42 – 5.79
BC4F1	2.69	2.53 – 2.84	2.21	2.11 – 2.33	2.74	2.48 – 2.94	2.52	2.31 – 2.71	15.61	14.7 – 16.92	5.13	3.71 – 5.75
BC5F1	2.75	2.66 – 2.79	2.36	2.27 – 2.40	2.79	2.66 – 2.89	2.57	2.46 – 2.66	15.09	13.39 – 16.38	5.04	4.07 – 5.43

Table 10 shows on a dry weight (DW) basis, the PMI protein concentrations in MZIR260 ranged from 2.53 to 2.84 µg/g DW in R6 kernels, and 2.48 to 2.94 µg/g DW in R6 senescence kernels, and 11.70 to 16.92 µg/g DW in R4 forage. On a fresh weight (FW) basis, the PMI protein concentrations ranged from 2.11 to 2.40 µg/g FW in R6 kernels, and 2.31 to 2.71 µg/g FW in R6 senescence kernels, and 3.42 to 5.79 µg/g FW in R4 forage.

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TABLE 11. Concentrations of eCry1Gb.1Ig and PMI Protein in Leaves from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis

Protein	Generation	Leaves (V6)				Leaves (R1)				Leaves (R6)			
		µg/g DW		µg/g FW		µg/g DW		µg/g FW		µg/g DW		µg/g FW	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
eCry1Gb.1Ig	BC2F1	62.76	50.05 – 80.22	4.09	2.06 – 6.50	219.25	177.68 – 327.90	49.71	43.87 – 60.38	103.25	90.51 – 121.79	43.84	32.79 – 55.92
eCry1Gb.1Ig	BC4F1	47.79	46.59 – 49.33	3.36	1.83 – 5.49	238.74	122.98 – 333.57	55.48	26.87 – 74.20	113.84	100.75 – 145.04	40.19	33.19 – 62.43
eCry1Gb.1Ig	BC5F1	66.60	49.87 – 79.05	4.06	2.59 – 6.42	258.04	200.41 – 345.56	63.51	42.74 – 72.47	108.41	88.40 – 162.82	41.55	27.89 – 83.99
PMI	BC2F1	10.71	8.13 – 12.05	0.68	0.42 – 1.03	18.52	16.56 – 20.79	4.32	3.83 – 5.86	8.63	5.84 – 9.58	3.76	2.13 – 4.71
PMI	BC4F1	8.42	7.18 – 10.04	0.59	0.32 – 1.02	16.64	14.78 – 18.64	3.87	3.33 – 4.43	11.77	7.54 – 19.22	3.99	1.98 – 5.94
PMI	BC5F1	10.51	9.91 – 11.35	0.65	0.37 – 0.95	17.29	13.90 – 19.74	4.29	2.97 – 6.10	8.82	7.06 – 10.05	2.86	1.82 – 4.09

Table 11 shows that the eCry1Gb.1Ig in MZIR260 ranged from 46.59 to 80.22 µg/g DW in V6 leaves, 122.98 to 345.56 µg/g DW in R1 leaves, 88.40 to 162.82 µg/g DW in R6 leaves; and on a fresh weight (FW) basis the eCry1Gb.1Ig in MZIR260 ranged from- 1.83 to 6.50 µg/g FW in V6 leaves, 26.87 to 74.20 µg/g FW in R1 leaves, 27.89 to 83.99 µg/g FW in R6 leaves. The ranges of PMI concentration on a DW basis measured in MZIR260 maize were 7.18 to 12.05 µg/g DW in V6 leaves, 13.90 to 20.79 µg/g DW in R1 leaves, 5.84 to 19.22 µg/g DW in R6 leaves; and on a FW were 0.32 to 1.03 µg/g FW in V6 leaves, 2.97 to 6.10 µg/g FW in R1 leaves, 1.82 to 5.94 µg/g FW in R6 leaves.

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TABLE 12. Concentrations of eCry1Gb.1Ig and PMI Protein in Roots from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis

Protein	Generation	Roots (V6)				Roots (R1)				Roots (R6)			
		µg/g DW		µg/g FW		µg/g DW		µg/g FW		µg/g DW		µg/g FW	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
eCry1Gb.1Ig	BC_2F_1	111.07	84.21 – 188.15	28.82	23.11 – 41.63	100.63	76.39 – 131.02	10.87	6.96 – 14.89	153.04	86.52 – 202.79	39.34	16.17 – 58.51
eCry1Gb.1Ig	BC_4F_1	89.11	77.15 – 113.25	19.74	10.29 – 27.09	111.18	77.04 – 159.57	13.46	8.81 – 19.35	145.09	70.61 – 223.52	52.30	26.83 – 87.31
eCry1Gb.1Ig	BC_5F_1	98.39	82.85 – 107.92	30.12	22.52 – 40.10	110.96	82.02 – 161.21	12.62	8.41 – 18.65	124.77	37.74 – 187.49	32.52	8.94 – 80.61
PMI	BC_2F1	26.89	24.07 – 31.79	7.21	5.77 – 8.60	5.41	3.29 – 8.49	0.56	0.37 – 0.84	2.28	1.57 – 3.52	0.60	0.20 – 1.11
PMI	BC_4F1	26.97	22.08 – 33.45	6.13	2.94 – 10.62	6.37	4.58 – 10.75	0.78	0.47 – 1.23	2.52	1.52 – 4.11	1.04	0.34 – 1.60
PMI	BC_5F1	33.48	26.60 – 38.47	10.14	8.69 – 13.77	6.21	4.51 – 8.70	0.71	0.46 – 0.98	1.87	1.15 – 2.49	0.54	0.21 – 1.07

Table 12 shows the range of eCry1Gb.1Ig concentrations on a dry weight (DW) basis in MZIR260 maize were 77.15 to 188.15 µg/g DW in V6 roots, 76.39 to 161.21 µg/g DW in R1 roots, 37.74 to 223.52 µg/g DW in R6 roots; and on a fresh weight (FW) basis eCry1Gb.1Ig concentrations ranged between 10.29 to 41.63 µg/g FW in V6 roots, 6.96 to 19.35 µg/g FW in R1 roots, 8.94 to 87.31 µg/g FW in R6 roots. The range of PMI concentration in MZIR260 maize were 77.15 to 188.15 µg/g DW in V6 roots, 76.39 to 161.21 µg/g DW in R1 roots, 37.74 to 223.52 µg/g DW in R6 roots; and on a FW basis the range of PMI concentration in MZIR260 maize were 2.94 to 13.77 µg/g FW in V6 roots, 0.37 to 1.23 µg/g FW in R1 roots, 0.20 to 1.60 µg/g FW in R6 roots.

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TABLE 13. Concentrations of eCry1Gb.1Ig and PMI Protein in R1 Pollen from MZIR260 Maize Plants of Multiple Generations on a DW and a FW Basis

Protein	Generation	Pollen (R1)			
		µg/g DW		µg/g FW	
		Mean	Range	Mean	Range
eCry1Gb.1Ig	BC_2F_1	337.44	–	185.93	–
eCry1Gb.1Ig	BC_4F_1	438.21	–	235.72	–
eCry1Gb.1Ig	BC_5F_1	291.04	–	77.14	–
PMI	BC_2F_1	36.79	–	20.27	–
PMI	BC_4F_1	35.88	–	19.30	–
PMI	BC_5F_1	43.27	–	11.47	–

Table 13 shows in R1 pollen the range of eCry1Gb.1Ig concentration across three generations on a dry weight (DW) basis measured in MZIR260 maize were 291.04 to 438.21 µg/g DW, and on a fresh weight (FW) basis measured 11.47 to 20.27 µg/g FW. In R1 pollen the ranges of PMI concentration across three generations on a DW basis measured in MZIR260 maize were 35.88 to 43.27 µg/g DW and on a FW basis measured 11.47 to 20.27 µg/g FW.

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(f) Conclusion – Molecular Characterisation

- Molecular characterization of MZIR260 maize confirmed that the maize genome contains a single copy of the MZIR260 insert, present at a single locus on chromosome 2.
- Genetic analysis demonstrated the integrity of the MZIR260 insert, absence of plasmid backbone including antibiotic resistance marker genes, and confirmed that integration of the insert into the maize genome did not disrupt any known maize endogenous genes.
- WGS and Mendelian inheritance analysis provided evidence of stable inheritance of the transgenes across multiple generations, also confirming the transgenes' stable integration within the nuclear genome.
- ELISA analysis confirmed stability of the phenotype over multiple generations.
- No evidence of hypothetical ORFs with biologically relevant sequence similarity to known or putative allergens or toxins of mammalian concern

Based on this information it can be concluded that no unintended changes leading to safety concerns were identified during the characterization of MZIR260 maize, and the transgenes *eCry1Gb.1Ig-03* and *pmi-15* exhibit stable expression in MZIR260 maize and follow Mendelian principles.

Section 2.04 Annex II References

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Article III. PART B. CHARACTERISATION AND SAFETY ASSESSMENT OF NEWLY EXPRESSED PROTEINS (NEPs)

Article III - Section 3.01 provides information to characterise the Newly Expressed Proteins (NEPs) - eCry1Gb.1Ig and PMI. The level and site of protein expression was included to determine potential exposure to these proteins through the consumption of MZIR260 maize, should had a particular hazard been identified.

A series of analytical characterisation studies describe the biochemical and functional equivalence of the proteins products *in planta* and in a recombinant *E.coli* expression system to confirm suitability as a surrogate in subsequent food and feed safety studies.

Post-translational glycosylation analysis was included to evaluate if the NEPs are expressed in MZIR260 as expected. Glycosylation, the post-translational modification process by which sugar molecules are attached to proteins, is linked to the initiation of the immunoglobulin E (IgE)-mediated allergic reactions (Altmann, 2007), and can enhance the stability of protein structures, thereby potentially increasing their resistance to enzymatic digestion (Pekar *et al*., 2018). This information can also be used as part of a weight of evidence approach to predict allergenicity.

Article III - Sections 3.02 and 3.03, describes a weight of evidence approach as outlined by the Codex Alimentarius Commission (Codex 2009), to evaluate the safety of the NEPs proteins derived from MZIR260 maize. This approach can include information on the source organisms, HOSU in foods, *in silico* comparisons to known and putative allergens and toxins of mammalian concern, digestive fate and stability under heat - mimicking the cooking process. The level of information provided to FSANZ was determined based on whether the protein had been evaluated in previous risk assessments.

- **Section 3.02** provides safety information for a protein considered identical to others previously assessed by FSANZ – PMI (A580; A1001 and A1060). The data in this current application consists of an updated bioinformatics comparison of the PMI amino acid sequence to known protein toxins, anti-nutrients and allergens
- **Section 3.03** evaluates the safety of the eCry1Gb.1Ig protein, a chimeric protein not reviewed by FSANZ to-date. In addition to the bioinformatics comparisons to known toxins and allergens, data is provided to show its stability to proteolysis in an appropriate gastrointestinal model, and its susceptibility to heat was provide.
- The source organisms and HOSU in foods is provided for both PMI and eCry1Gb.1Ig.

The updated bioinformatic comparisons did not show similarity to biologically relevant toxins. Both eCry1Gb.1Ig and PMI are considered susceptible to proteolysis. Therefore, animal toxicity studies were not warranted.

Article III - Section 3.05 provides information on the compositional analysis, to show that no unintended changes in composition had occurred in the grain and forage derived from MZIR260 maize as a result of the transformation process itself or the introduced NEPs.

The components in MZIR260 maize forage and grain were analysed according to OECD guidelines. Comparisons to reference and/or literature ranges were made to determine the range of

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natural variation and to establish the *biological significance* of any identified statistical differences. Through this process it was determined that forage and grain from Event MZIR260 are not materially different in nutrient composition to conventional maize. Based on the outcomes of this analysis no further nutritional impact assessments were conducted.

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Section 3.01 Characterization of the NEPs - eCry1Gb.1Ig and PMI

(a) Expression levels of eCry1Gb.1Ig and PMI proteins in the MZIR260 F_1 generation across in several tissue types, developmental stages, and field environments

A study using enzyme-linked immunosorbent assay (ELISA) to provide a profile of PMI and eCry1Gb.1Ig protein concentrations present in MZIR260 maize in several tissue types, developmental stages and across four different field environments is provided as part of this application dossier (Appendix 16).

Appendix 16. Bednarcik, Mark. RIR-0006800. (2023). *Quantification of eCry1Gb.1Ig, and Phosphomannose Isomerase in Event MZIR260 Maize Tissues. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

Enzyme-linked immunosorbent assays (ELISA) were used to quantify the transgenic proteins in each maize tissue sample. Inclusion of nontransgenic, near- isogenic maize tissue extracts confirmed the absence of matrix effects for the applied analytical method. Tables 14-16 provided in this application dossier include *all* location data. The concentrations of the protein for each individual location (L01: Germansville, PA; L02: York, NE; L03: Richland, I and L04: Stewardson, IL) can be found in the study report provided - Appendix 16.

In summary, MZIR260 F_1 maize and the corresponding nontransgenic, near-isogenic maize hybrid was grown at four field trial locations in the United States in 2022. Each field trial was designed to generate tissue samples from field-grown maize plants cultivated in accordance with common agricultural practices. The geographic locations selected for the field trials were representative of the agricultural environment where this variety of maize would typically be grown. At each location, one plot was planted with MZIR260 maize, and another plot was planted with nontransgenic, near-isogenic maize. From each plot, pollen and forage were collected from maize plants at one growth stage (R1), kernels were collected from two different growth stages (R6 and R6 senescence), whole plants were collected from maize plants at three different growth stages (V6, R1, R6) and leaves and roots were collected from maize plants at four different growth stages (V6, R1, R6 and R6 senescence). Five replicate samples of each tissue type, except pollen, were collected from the test plot, and two samples from the control plot were collected. For pollen, one pooled sample from 20 tassels was collected from the test and one pooled sample from 20 tassels was collected from the control plot.

Kernels from MZIR260 maize, are most likely tissue to enter the food and feed supply chains, as either grain or grain by-products or forage.

- Table 14 shows the range of protein concentrations for both eCry1Gb.1Ig and PMI as observed in MZIR260 maize kernels (grain) and forage, on a dry-weight (DW) and fresh-weight (FW) basis.
- Table 15 shows the range of protein concentrations for eCry1Gb.1Ig as observed in MZIR260 maize leaves, roots, whole plants, and pollen on a dry-weight (DW) and fresh-weight (FW) basis for maize.

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- Table 16 shows the range of protein concentrations PMI, as observed in MZIR260 maize leaves, roots, whole plants, and pollen on a dry-weight (DW) and fresh-weight (FW) basis for maize.

In Conclusion:

- The data provided in this study provided a profile of eCry1Gb.1Ig and PMI protein concentrations present in MZIR260 maize in various tissue types and developmental stages across four different field environments.
- Concentrations of eCry1Gb.1Ig and PMI were determined in all MZIR260 maize tissue types analysed.

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TABLE 14. Concentrations of eCry1Gb.1Ig and PMI in Kernels and Forage of MZIR260 Grown Across ***All Locations*** and Growth Stages on a Dry-Weight and a Fresh-Weight Basis

			µg/g DW		µg/g FW	
Protein	**Stage [a,b]**	**Tissue**	**Mean± SD**	**Range**	**Mean ± SD**	**Range**
eCry1Gb.1Ig	R6 (BBCH 87)	Kernel	297 ± 42.3	221 – 368	195 ± 25.0	152 – 251
eCry1Gb.1Ig	R6 Senescence (BBCH 99)	Kernel	262 ± 48.9	168 – 347	206 ± 39.4	142 – 287
eCry1Gb.1Ig	R4 (BBCH 85)	Forage	274 ± 71.0	125 – 474	93.6 ± 20.0	33.3–160
PMI	R6 (BBCH 87)	Kernel	10.1 ± 2.75	6.08 – 16.0	6.63 ± 1.73	4.47 – 10.4
PMI	R6 Senescence (BBCH 99)	Kernel	6.73 ± 1.42	4.05 – 12.3	5.17 ± 1.07	3.37 – 7.94
PMI	R4 (BBCH 85)	Forage	11.1 ± 2.12	<LOD – 16.1	3.67 ± 0.675	< LOD - 5.48

[a] V–R scale (Abendroth et al. 2011).
[b]BBCH = Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale (Meier 2001)

Table 14 shows the range of protein concentrations for both eCry1Gb.1Ig and PMI as observed in MZIR260 maize kernels (grain) and forage, on a dry-weight (DW) and fresh-weight (FW) basis. As highlighted in green, the highest mean concentration of eCry1Gb.1Ig in edible portion (kernels or forage as food or feed) was the R6 kernels with 297 ± 42.3 ug/g on a dry weight basis and for PMI the highest mean concentration in the edible portion of the crop was forage with 11.1 ± 2.12 ug/g on a dry weight basis. In kernels, across two growth stages the concentration of eCry1Gb.1Ig ranged from 168 to 368 µg/g DW and from 142 to 287 µg/g FW and the PMI concentration ranged from 4.05 to 16.0 µg/g DW and from 3.37 to 10.4 µg/g FW. In forage, the concentration of eCry1Gb.1Ig across a single growth stage ranged from 125 to 474 µg/g DW and from 33.3 to 160 µg/g FW and the PMI concentration ranged from less than LOD to 16.1 µg/g DW and from less than LOD to 5.48 µg/g FW. The blue shading shows the highest and lowest number in the range per growth stage.

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TABLE 15. Concentrations of eCry1Gb.1Ig in Leaves, Root, Whole Plants and Pollen of MZIR260 Grown ***Across All*** Locations and Growth Stages on a Dry-Weight and a Fresh-Weight Basis

			µg/g DW		µg/g FW	
Protein	**Stage [a,b]**	**Tissue**	**Mean± SD**	**Range**	**Mean ± SD**	**Range**
eCry1Gb.1Ig	V6 (BBCH 16)	Leaves	233 ± 78.7	64.7 – 492	35.5 ± 12.2	7.62 –77.1
eCry1Gb.1Ig	R1(BBCH 63-65)	Leaves	266 ± 80.4	95.7 – 429	65.4 ± 19.9	24.4 – 107
eCry1Gb.1Ig	R6 (BBCH 87)	Leaves	681 ± 163	339 – 1140	307 ± 73.5	123 – 577
eCry1Gb.1Ig	R6 Senescence (BBCH 99)	Leaves	490 ± 175	144 – 1370	324 ± 118	133 – 891
eCry1Gb.1Ig	V6 (BBCH 16)	Roots	380 ± 73.2	215 – 758	44.3 ± 8.72	20.9 – 85.5
eCry1Gb.1Ig	R1(BBCH 63-65)	Roots	379 ± 56.7	158 – 478	54.8 ± 10.7	21.7–86.8
eCry1Gb.1Ig	R6 (BBCH 87)	Roots	535 ± 96.7	297 – 905	94.4 ± 16.6	41.6 – 181
eCry1Gb.1Ig	R6 Senescence (BBCH 99)	Roots	547 ± 107	305 – 747	101 ± 16.8	60.2 – 164
eCry1Gb.1Ig	V6 (BBCH 16)	Whole Plants	215 ± 46.9 8	89.8 – 378	24.9 ± 6.27	5.70 – 40.2
eCry1Gb.1Ig	R1(BBCH 63-65)	Whole Plants	198 ± 76.0	93.8 – 341	40.2 ± 15.3	18.8–65.4
eCry1Gb.1Ig	R6 (BBCH 87)	Whole Plants	324 ± 68.0	202 – 482	181 ± 38.7	97.5–282
eCry1Gb.1Ig	R1(BBCH 63-65)	Pollen	773 ± 50.91	699 – 816	463 ± 94.92	378 – 582

[a] V–R scale (Abendroth et al. 2011).
[b]BBCH = Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale (Meier 2001)

Table 15 shows the range of protein concentrations for eCry1Gb.1Ig as observed in MZIR260 leaves, roots, whole plants, and pollen on a dry-weight (DW) and fresh-weight (FW) basis. In leaves, the concentration of eCry1Gb.1Ig across four growth stages ranged from 64.7 to 1370 µg/g DW and ranged from 7.62 to 891 µg/g FW. In roots, the concentration of eCry1Gb.1Ig across four growth stages ranged from 158 to 905 µg/g DW and ranged from 20.9 to 181 µg/g FW. In whole plants, the concentration of eCry1Gb.1Ig across three growth stages ranged from 89.8 to 482 µg/g DW in whole plants and ranged from 5.7 to 282 µg/g FW. In pollen, the concentration of eCry1Gb.1Ig ranged from 699 to 816 µg/g DW and ranged from 378 to 582 µg/g FW. The blue shading shows the highest and lowest number in the range per growth stage.

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TABLE 16. Concentrations of PMI in Leaves, Root, Whole Plants and Pollen of MZIR260 Grown Across *All Locations* and Growth Stages on a Dry-Weight and a Fresh-Weight Basis

			µg/g DW		µg/g FW	
Protein	**Stage [a,b]**	**Tissue**	**Mean± SD**	**Range**	**Mean ± SD**	**Range**
PMI	V6 (BBCH 16)	Leaves	20.5 ± 2.12	15.1 – 25.7	3.08 ± 0.348	1.78 – 4.06
PMI	R1(BBCH 63-65)	Leaves	13.3 ± 2.67	9.25 – 20.0	3.28 ± 0.686	2.17 – 4.74
PMI	R6 (BBCH 87)	Leaves	16.1 ± 6.64	3.24 – 33.7	6.54 ± 2.33	1.57 – 11.8
PMI	R6 Senescence (BBCH 99)	Leaves	4.78 ± 2.34	<LOD – 14.3	4.03 ± 1.94	<LOD – 13.3
PMI	V6 (BBCH 16)	Roots	18.6 ± 3.35	9.40 – 30.8	2.12 ± 0.408	1.15 – 3.04
PMI	R1(BBCH 63-65)	Roots	7.03 ± 1.16	4.79 – 9.88	1.01 ± 0.197	0.658 – 1.57
PMI	R6 (BBCH 87)	Roots	9.03 ± 2.08	3.81 – 17.8	1.60 ± 0.393	0.530 – 3.35
PMI	R6 Senescence (BBCH 99)	Roots	5.95 ± 1.19	2.45 – 10.7	1.14 ± 0.266	0.459 – 2.53
PMI	V6 (BBCH 16)	Whole Plants	21.0 ± 2.15	12.8 – 31.5	2.42 ± 0.399	0.712 – 4.06
PMI	R1(BBCH 63-65)	Whole Plants	13.2 ± 1.40	8.76 – 16.6	2.69 ± 0.354	1.73 – 4.18
PMI	R6 (BBCH 87)	Whole Plants	8.57 ± 2.42	4.24 – 14.0	4.67 ± 1.10	2.77 – 6.70
PMI	R1(BBCH 63-65)	Pollen	17.6 ± 14.65	9.93 – 39.6	12.4 ±13.78	4.77 – 33.0

[a] V–R scale (Abendroth et al. 2011).
[b]BBCH = Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale (Meier 2001)

Table 16 shows the range of protein concentrations for PMI as observed in MZIR260 leaves, roots, whole plants, and pollen on a dry-weight (DW) and fresh-weight (FW) basis. In leaves, the concentration of PMI across four growth stages ranged from less than the limit of detection (LOD) to 33.7 µg/g DW and ranged from less than LOD to 13.3 µg/g FW. In roots, the concentration of PMI across four growth stages ranged from 2.45 to 30.8 µg/g DW and ranged from 0.459 to 3.35 µg/g FW. In whole plants, the concentration of PMI across three growth stages ranged from 4.24 to 31.5 µg/g DW and ranged from 0.712 to 6.70 µg/g FW. In pollen, the concentration of PMI ranged from 9.93 to 39.6 µg/g DW and ranged from 4.77 to 33.0 µg/g FW. The blue shading shows the highest and lowest number in the range per growth stage.

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(b) Equivalence of the eCry1Gb.1Ig protein expressed *in planta* and a microbial system

To assess the biochemical and functional equivalence of the eCry1Gb.1Ig protein produced in Event MZIR260 maize plants and eCry1Gb.1Ig protein produced in a recombinant *Escherichia coli* expression system, the proteins from both sources were compared with respect to insecticidal activity, apparent molecular weight, immunoreactivity, peptide mass coverage, and glycosylation status (Appendix 17).

The test substance 'ECRY1GB.1IG-0121' used in these experiments is described in (Appendix 18).

Both study reports are provided as part of this application dossier.

Appendix 17. Ellur, Vishnutej and Wu, Jianhong. TK0549521. (2024). *Comparison of eCry1Gb.1Ig Protein Produced in Recombinant Escherichia coli with eCry1Gb.1Ig Protein Produced in Event MZIR260 Derived Maize Plants. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

Appendix 18. Luo Guoling. (2022). *Characterization of Microbially Produced Test Substance ECRY1GB.1IG-0121 Containing ecry1Gb.1Ig Protein.* Unpublished. Syngenta Crop Protection, LLC.

(i) Functional activity of eCry1Gb.1Ig

The insecticidal activities of microbially-produced eCry1Gb.1Ig protein, eCry1Gb.1Ig protein in MZIR260 maize leaf crude extract, and nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein were determined in three independent diet surface bioassays against first instar soybean looper (SBL) larvae (Appendix 17: Section 3.9 & 4.5; Pages 17, 23 & 24).

In summary, the eCry1Gb.1Ig protein contained in the microbially-produced test substance was solubilized in 50 mM CAPS, pH 10.5, 1 mM TCEP buffer, and the appropriate amount was prepared according to the amount determined by the ELISA analyses of MZIR260 maize leaf extracts, and further dilutions were made in 50 mM CAPS, pH 10.5, 1 mM TCEP. Test and control solutions of MZIR260 maize leaf crude extract, nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein, and nontransgenic maize leaf crude extract were prepared to have an equivalence in total protein. The MZIR260 maize leaf crude extract and nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein samples were diluted in 50 mM CAPS, pH 10.5, 1 mM TCEP. The dilutions resulted in a series of test solutions with eCry1Gb.1Ig protein concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156, and 0.0078 µg/ml, respectively, for each eCry1Gb.1Ig protein-containing sample. Nontransgenic maize leaf crude extracts, with total protein concentrations equivalent to the 1 µg/ml eCry1Gb.1Ig protein-containing MZIR260 maize leaf crude extract sample and the nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein, were diluted in 50 mM CAPS, pH 10.5, 1 mM TCEP were included as negative controls.

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Bioassays were conducted in 24-well culture plates, one culture plate per protein concentration. Each well contained 800 µl of artificial diet, overlaid with 50 µl of the above individual test or control solution after the diet solidified. Each well was infested with one SBL larva. Plates were sealed with clear polyolefin tape and incubated in a controlled condition of 22°C ± 5°C, with a 14 (hr) hour/10 hour (hr) light/dark cycle. Mortality rates were assessed daily starting from 72 hours until 168 hours after infestation. Bioactivity data from the three independent bioassays at the 168 hours endpoint were combined and used to report total mortality and generate a 50% lethal concentration (LC_{50}) value for each respective treatment. The dilution buffer alone was used as the bioassay negative control for probit analysis.

The insecticidal activities of microbially-produced eCry1Gb.1Ig protein, MZIR260 maize leaf crude extract, and nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein have similar response ranges and slopes. The microbially-produced eCry1Gb.1Ig protein gave an LC_{50} of 2.42 ng/cm^2, with a 95% confidence interval (CI) of 1.98 to 2.9 ng/cm^2, which was very similar to that of the MZIR260 maize leaf crude extract (2.84 ng/cm^2, with a 95% CI of 2.33 to 3.38 ng/cm^2) and the nontransgenic maize crude leaf extract fortified with microbially-produced eCry1Gb.1Ig protein (2.69 ng/cm^2, with a 95% CI of 2.23 to 3.18 ng/cm^2), with overlapping 95% confidence intervals. The composite results of the three independent insect bioassays are summarized in Table 17.

In Conclusion:

- Composite 168-hour LC_{50} estimates from the bioassays were 2.42 ng/cm^2 for the microbially-produced eCry1Gb.1Ig protein in the absence of nontransgenic leaf extract, 2.69 ng/cm^2 for the microbially-produced eCry1Gb.1Ig protein in the presence of nontransgenic leaf extract, and 2.84 ng/cm^2 for the plant-produced eCry1Gb.1Ig protein.

- Insecticidal activity bioassays against first instar larvae of the soybean looper (SBL, *Chrysodeixis includens*) revealed comparable 50% lethal concentration (LC_{50}) values.

- Microbially-produced eCry1Gb.1Ig protein is functionally equivalent to the plant-produced eCry1Gb.1Ig protein.

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TABLE 17. Estimated LC_{50} for eCry1Gb.1Ig in MZIR260 Maize Crude Leaf Extract, Microbially Produced eCry1Gb.1Ig, and Nontransgenic Maize Crude Extract Fortified with Microbially-produced eCry1Gb.1Ig (168 Hours)

Treatment	LC_{50} (ng/cm^2)	95% CI (ng/cm^2)	Slope ± SEM[a]	95% CI
Microbially-produced eCry1Gb.1Ig protein	2.42	1.98–2.9	1.88 ± 0.152	1.58–2.18
eCry1Gb.1Ig protein in MZIR260 maize leaf crude extract	2.84	2.33–3.38	2.14 ± 0.191	1.77–2.51
Nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein	2.69	2.23–3.18	2.25 ± 0.196	1.87–2.63

[a]SEM = standard error of the mean.

(ii) Immunoreactivity and molecular weight determination of eCry1Gb.1Ig

The apparent molecular weight and immunoreactivity of both microbially- and plant-produced eCry1Gb.1Ig protein were investigated using Western blot analysis (Appendix 17: Section 3.4 & 4.2; Pages 14, 18 & 19).

In summary, the analysis included microbially produced eCry1Gb.1Ig protein, purified plant-produced eCry1Gb.1Ig protein, MZIR260 maize leaf crude extract, nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein, and a nontransgenic maize leaf crude extract as a negative control. A nontransgenic maize leaf crude extract sample fortified with microbially-produced eCry1Gb.1Ig protein was included in the Western blot analysis to determine the plant matrix interference. Samples were prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a 4-12% Bolt™ Bis-Tris gel and Bolt™ 2-(*N*-morpholino) ethanesulfonic acid (MES) running buffer. The molecular weight standard was the Invitrogen™ SeeBlue® Plus2 pre-stained standard. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane via electroblotting. The membrane was probed with a polyclonal goat antibody capable of binding to eCry1Gb.1Ig protein. Detection of eCry1Gb.1Ig protein was accomplished through the binding of polyclonal donkey anti-goat antibodies conjugated with alkaline phosphatase enzyme, which catalyzed the conversion of the chromogenic substrate solution BCIP®/NBT. The blot was imaged using a Bio-Rad GS-900 densitometer optical scanner. The Western blot was examined for the presence of intact immunoreactive eCry1Gb.1Ig protein or other immunoreactive eCry1Gb.1Ig-derived fragments.

Western blot analysis revealed immunoreactive bands corresponding to the anticipated molecular weight of ~ 133 kDa of eCry1Gb.1Ig protein for all the samples except for the nontransgenic maize leaf crude extract (Figure 15, Lanes 2 through 5). No immunoreactive bands were observed for the nontransgenic maize leaf crude extract (Figure 15, Lane 6). There was a very faint protein band of ~49 kDa for the microbially-produced eCry1Gb.1Ig protein, purified plant-produced eCry1Gb.1Ig protein, MZIR260 maize leaf crude extract, and nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb.1Ig protein (Figure 15, Lanes 2 through 5). Since these protein bands cross-reacted with eCry1Gb.1Ig-specific antibody, and none of these bands were

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observed in nontransgenic maize leaf crude extract (Figure 15, Lane 6), the ~49 kDa band is likely a degraded eCry1Gb.1Ig protein fragments. The bands greater than 198 kDa are likely multimers of the eCry1Gb.1Ig protein. The protein aggregation (dark smear) identified at the top of the blot greater than 198 kDa molecular weight marker for plant-derived proteins, i.e., purified plant-produced eCry1Gb.1Ig protein (Figure 15, Lane 3) and MZIR260 maize leaf crude extract (Figure 15, Lane 4), is most likely due to plant matrix interference.

In Conclusion:

- Western blot analysis of microbially-produced eCry1Gb.1Ig protein in the presence or absence of nontransgenic leaf extract and plant-produced eCry1Gb.1Ig proteins showed identical mobility consistent with the predicted molecular weight of approximately 133 kilodaltons.
- Western blot analysis shows that the identity and integrity of microbially-produced eCry1Gb.1Ig protein and plant-produced eCry1Gb.1Ig protein are as expected.
- Both microbial and plant derived proteins cross-reacted with the same eCry1Gb.1Ig-specific antibody, confirming similar immunoreactivity.

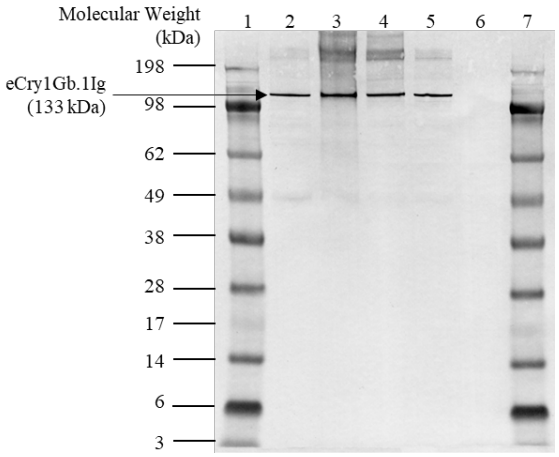

FIGURE 15. Western Blot Analysis of the Microbially Produced and Plant-Produced eCry1Gb.1Ig

Lane 1: Molecular weight standard
Lane 2: Microbially-produced eCry1Gb.1Ig (6 ng eCry1Gb.1Ig)
Lane 3: eCry1Gb.1Ig protein purified from MZIR260 maize (6 ng eCry1Gb.1Ig)
Lane 4: MZIR260 maize leaf crude extract (6 ng eCry1Gb.1Ig; 5.94 µg total protein)
Lane 5: Nontransgenic maize leaf crude extract fortified with microbially-produced eCry1Gb (6 ng eCry1Gb.1Ig; 5.94 µg total protein)
Lane 6: Nontransgenic maize leaf crude extract (5.94 µg total protein)
Lane 7: Molecular weight standard

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(iii) Amino acid sequence of eCry1Gb.1Ig

```
   1  MEINNQNQCV PYNCLNNPES EILNVAIFSS EQVAEIHLKI TRLILENFLP
  51  GGSFAFGLFD LIWGIFNEDQ WSAFLRQVEE LINQRITEFA RGQAIQRLVG
 101  FGRSYDEYIL ALKEWENDPD NPASKERVRT RFRTTDDALL TGVPLMAIPG
 151  FELATLSVYA QSANLHLALL RDAVFFGERW GLTQTNINDL YSRLKNSIRD
 201  YTNHCVRFYN IGLGNLNVIR PEYYRFQREL TISVLDLVAL FPNYDIRTYP
 251  IPTKSQLTRE IYTDPIISPG AQAGYTLQDV LREPHLMDFL NRLIIYTGEY
 301  RGIRHWAGHE VESSRTGMMT NIRFPLYGTA ATAEPTRFIT PSTFPGLNLF
 351  YRTLSAPIFR DEPGANIIIR YRTSLVEGVG FIQPNNGEQL YRVRGTLDSL
 401  DQLPLEGESS LTEYSHRLCH VRFAQSLRNA EPLDYARVPM FSWTHRSATP
 451  TNTIDPDVIT QIPLVKAHTL QSGTTVVKGP GFTGGDILRR TSGGPFAFSN
 501  VNLDWNLSQR YRARIRYAST TNLRMYVTIA GERIFAGQFN KTMNTGDPLT
 551  FQSFSYATID TAFTFPTKAS SLTVGADTFS SGNEVYVDRF ELIPVTATFE
 601  AEYDLEKAQK AVNALFTSSN QIGLKTDVTD YHIDKVSNLV ECLSDEFCLD
 651  EKRELSEKVK HAKRLCDERN LLQDPNFRGI NRQPDRGWRG STDITIQGGD
 701  DVFKENYVTL PGTFDECYPT YLYQKIDESK LKAYTRYELR GYIEDSQDLE
 751  IYLIRYNAKH ETVNVPGTGS LWPLSAQSPI GKCGEPNRCA THLEWNPDLD
 801  CSCRDGEKCA HHSHHFSLDI DVGCTDLNED LGVWVIFKIK TQDGHARLGN
 851  LEFLEEKPLV GEALARVKRA EKKWRDKREK LELETNIVYK EAKKSVDALF
 901  VNSQYDRLQA DTNIAIIHAA DKRVHSIREA YLPELSVIPG VNAAIFEELE
 951  GRIFTAYSLY DARNVIKNGD FNNGLSCWNV KGHVDVEEQN NHRSVLVVPE
1001  WEAEVSQEVR VCPGRGYILR VTAYKEGYGE GCVTIHEIED NTDELKFSNC
1051  VEEEIYPNNT VTCNDYTATQ EEYEGTYTSR NRGYDGAYES NSSVPADYAS
1101  AYEEKAYTDG RRDNTCESNR GYGDYTPLPA GYVTKELEYF PETDKVWIEI
1151  GETEGTFIVD SVELLLMEE
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FIGURE 16. Amino Acid (Deduced) Sequence of the eCry1Gb.1Ig Protein

(iv) Peptide mass coverage analysis of eCry1Gb.1Ig

Peptide mass coverage analysis was used to determine the identity of the microbially-produced eCry1Gb.1Ig protein and the purified plant-produced eCry1Gb.1Ig protein (Appendix 17: Sections 3.5 - 3.7 & 4.3 pages 14-16; 19-21).

In summary, proteolytic peptides for peptide mass coverage analysis of purified plant-produced eCry1Gb.1Ig protein were produced using an in-gel protein digestion. Protein bands corresponding to the molecular weight of eCry1Gb.1Ig protein were excised from the SDSPAGE gel lanes containing purified plant-produced eCry1Gb.1Ig protein. Reduced and alkylated samples were washed with water, dehydrated in acetonitrile, dried, and rehydrated on ice in the 50 mM TEAB buffer containing 1µg of either trypsin/LysC mixture or chymotrypsin.

Proteolytic peptides for peptide mass coverage analysis of microbially-produced eCry1Gb.1Ig protein were produced by digesting the proteins bound to S-Trap™ resin (ProtiFi LLC, Fairport, NY). Reduced and alkylated samples was digested with 3.45 µg of trypsin/LysC mixture as well as 3.45 µg of chymotrypsin in a separate digestion reaction. The digestions were carried out at 47°C for 1 hour and 30 minutes.

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The samples were injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS).

Each acquired MS/MS spectrum was analyzed using MaxQuant software, version 2.4.0.0 (Cox and Mann, 2008) to obtain the peptide identities.

The system suitability spectra from BSA digest analysis were searched against *Bos taurus* reference proteome (UniProt, 2023a). The spectra of microbially-produced eCry1Gb.1Ig protein were searched against the databases containing eCry1Gb.1Ig protein amino acid sequence and *E. coli* strain K12 reference proteome (UniProt., 2023b). The spectra of the purified plant-produced eCry1Gb.1Ig protein were searched against the databases containing eCry1Gb.1Ig protein amino acid sequence and maize reference proteome (internal database). Only peptides with a false discovery rate of less than 1% were considered identified.

The collective analysis of the two proteolytic digests (trypsin and chymotrypsin) for the microbially-produced eCry1Gb.1Ig resulted in coverage of 96% of the total predicted eCry1Gb.1Ig amino acid sequence (Figure 17). The collective analysis of the two proteolytic digests (trypsin and chymotrypsin) for the purified eCry1Gb.1Ig preparation from MZIR260 maize leaf extract yielded coverage of 88% of the total predicted eCry1Gb.1Ig amino acid sequence (Figure 18).

In Conclusion:

- Peptide mass coverage analysis of the microbially- and the plant-produced eCry1Gb.1Ig proteins resulted in 96% and 88% coverage of the predicted eCry1Gb.1Ig protein amino acid sequence, respectively.

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1	MEINNQNQCV	PYNCLNNPES	EILNVAIFSS	EQVAEIHLK**I**	**TRLILENFLP**	50
51	**GGSFAFGLFD**	**LIW**GIFNEDQ	WSAFLRQVEE	LINQR**ITEFA**	**R**GQAIQRLVG	100
101	FGRSYDEYIL	ALKEWENDPD	NPASKER**VRT**	**R**FRTTDDALL	TGVPLMAIPG	150
151	FELATLSVYA	QSANLHLALL	RDAVFFGERW	GLTQTNINDL	YSRLKNSIRD	200
201	YTNHCVRFYN	IGLGNLNVIR	PEYYRFQREL	TISVLDLVAL	FPNYDIRTYP	250
251	IPTKSQLTRE	IYTDPIISPG	AQAGYTLQDV	LREPHLMDFL	NRLIIYTGEY	300
301	RGIRHWAGHE	VESSRTGMMT	NIRFPLYGTA	ATAEPTRFIT	PSTFPGLNLF	350
351	YRTLSAPIFR	DEPGANIIIR	YRTSLVEGVG	FIQPNNGEQL	YRVRGTLDSL	400
401	DQLPLEGESS	LTEYSHRLCH	VRFAQSLRNA	EPLDYARVPM	FSWTHRSATP	450
451	TNTIDPDVIT	QIPLVKAHTL	QSGTTVVKGP	GFTGGDILRR	TSGGPFAFSN	500
501	VNLDWNLSQR	YRARIRYAST	TNLRMYVTIA	GERIFAGQFN	KTMNTGDPLT	550
551	FQSFSYATID	TAFTFPTKAS	SLTVGADTFS	SGNEVYVDRF	ELIPVTATFE	600
601	AEYDLEKAQK	AVNALFTSSN	QIGLKTDVTD	YHIDKVSNLV	ECLSDEFCLD	650
651	EKRELSEK**VK**	**HAK**RLCDERN	LLQDPNFRGI	NRQPDRGWRG	STDITIQGGD	700
701	DVFKENYVTL	PGTFDECYPT	YLYQKIDESK	LKAYTRYELR	GYIEDSQDLE	750
751	IYLIRYNAKH	ETVNVPGTGS	LWPLSAQSPI	GKCGEPNRCA	THLEWNPDLD	800
801	CSCRDGEKCA	HHSHHFSLDI	DVGCTDLNED	LGVWVIFK**IK**	TQDGHARLGN	850
851	LEFLEEKPLV	GEALARVK**RA**	**EKKWRDK**REK	LELETNIVYK	EAKKSVDALF	900
901	VNSQYDRLQA	DTNIAIIHAA	DKRVHSIREA	YLPELSVIPG	VNAAIFEELE	950
951	GRIFTAYSLY	DARNVIKNGD	FNNGLSCWNV	KGHVDVEEQN	NHRSVLVVPE	1000
1001	WEAEVSQEVR	VCPGRGYILR	VTAYKEGYGE	GCVTIHEIED	NTDELKFSNC	1001
1051	VEEEIYPNNT	VTCNDYTATQ	EEYEGTYTSR	NRGYDGAYES	NSSVPADYAS	1050
1101	AYEEKAYTDG	RRDNTCESNR	GYGDYTPLPA	GYVTKELEYF	PETDKVWIEI	1100
1151	GETEGTFIVD	SVELLLMEE				1169

Underlined indicates amino acids were confirmed; bold and not underlined indicates amino acids were not confirmed.

FIGURE 17. Amino Acid Sequence Coverage Map for Microbially-produced eCry1Gb.1Ig

1	MEINNQNQCV	PY**NCL**NNPES	EILNVAIFSS	EQVAEIHLK**I**	**TRL**ILENFLP	50
51	GGSF**AFGLFD**	**LIW**GIFNEDQ	WSAFLRQVEE	LINQRITEF**A**	**RGQAIQRLVG**	100
101	**FGR**SYDEYIL	ALKEWENDPD	NPASK**ERVRT**	**RFR**TTDDALL	TGVPLMAIPG	150
151	FELATLSVYA	QSANLHLALL	RDAVFFGERW	GLTQTNINDL	YSR**L**KNSIRD	200
201	YTNHCVRFYN	IGLGNLNVIR	PEYYR**FQR**EL	TISVLDLVAL	FPNYDIRTYP	250
251	IPTKSQLTRE	IYTDPIISPG	AQAGYTLQDV	LREPHLMDFL	NRLIIYTGEY	300
301	R**GIR**HWAGHE	VESSRTGMMT	NIRFPLYGTA	ATAEPTRFIT	PSTFPGLNLF	350
351	YRTLSAPIFR	DEPGANIIIR	YRTSLVEGVG	FIQPNNGEQL	YRVRGTLDSL	400
401	DQLPLEGESS	LTEYSHR**LCH**	**VRFAQSLR**NA	EPLDYARVPM	FSWTHRSATP	450
451	TNTIDPDVIT	QIPLVKAHTL	QSGTTVVKGP	GFTGGDILRR	TSGGPFAFSN	500
501	VNLDWNLSQR	**YRARIR**YAST	TNLRMYVTIA	GERIF**AGQF**N	KTMNTGDPLT	550
551	FQSFSYATID	TAFTFPTKAS	SLTVGADTFS	SGNEVYVDRF	ELIPVTATFE	600
601	AEYDLEK**AQK**	AVNALFTSSN	QIGLKTDVTD	YHIDKVSNLV	ECLSDEFCLD	650
651	EKREL**SEKVK**	**HAKRL**CDERN	LLQDPNFRGI	NRQPDRGWRG	STDITIQGGD	700
701	DVFKENYVTL	PGTFDECYPT	YLYQKIDESK	L**KAYTRY**ELR	GYIEDSQDLE	750
751	IYLIR**Y**NAKH	ETVNVPGTGS	LWPLSAQSPI	GK**CGEPNR**CA	THLEWNPDLD	800
801	CSCR**DGEKCA**	**HHSHHF**SLDI	DVGCTDLNED	LGVW**VIF**KIK	TQDGHARLGN	850
851	LEFLEEKPLV	GEALAR**VKRA**	**EKKW**RDKREK	LELETNIVYK	EAKKSVDALF	900
901	VNSQYDRLQA	DTNIAIIHAA	DKR**VHSIR**EA	YLPELSVIPG	VNAAIFEELE	950
951	GRIFTAYSLY	DAR**NVIK**NGD	FNNGLSCWNV	KGHVDVEEQN	NHRSVLVVPE	1000
1001	WEAEVSQEVR	VCPGRGY**ILR**	**VTAY**KEGYGE	GCVTIHEIED	NTDELKFSNC	1050
1051	VEEEIYPNNT	VTCNDYTATQ	EEYEGTYTSR	NRGYDGAYES	NSSVPADYAS	1100
1101	AYEEKAYTDG	RRDNTCESNR	GYGDYTPLPA	GYVTKELEYF	PETDKVW**IEI**	1150
1151	**GETEGTF**IVD	SVELLLMEE				1169

Underlined indicates amino acids were confirmed; bold and not underlined indicates amino acids were not confirmed.

FIGURE 18. Amino Acid Sequence Coverage Map for Plant-produced eCry1Gb.1Ig

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(v) N-terminal and C-terminal peptide sequencing of eCry1Gb.1Ig

In addition to overall amino acid sequence coverage, the N-terminal peptide sequences and C-terminal peptide sequences were identified by peptide mass coverage analysis for both the microbially- and purified plant-produced eCry1Gb.1Ig proteins and compared to the predicted eCry1Gb.1Ig protein amino acid sequence (Figures 19 and 20) finding that the N-terminal peptide and the C-terminal peptide sequences of both microbially- and plant-produced eCry1Gb.1Ig proteins were identical to each other and consistent with the predicted sequence (Appendix 17: Page 21-22).

Amino acid position	1	2	3	4	5	6	7	8	9	10	11	12
Predicted eCry1Gb.1Ig protein sequence[a]	M	E	I	N	N	Q	N	Q	C	V	P	Y
Microbially-produced eCry1Gb.1Ig[b] protein	M	E	I	N	N	Q	N	Q	C	V	P	Y
Purified plant-produced eCry1Gb.1Ig[b] protein	M	E	I	N	N	Q	N	Q	C	V	P	Y

[a]Predicted sequence deduced from the nucleotide sequence.
[b]The N-terminal peptide for both the microbially-produced eCry1Gb.1Ig and purified plant-produced eCry1Gb.1Ig protein was identified from the chymotrypsin digest.

FIGURE 19. Amino Acid Sequence Analysis of N-Terminal Peptides of Microbially-produced and Plant-produced eCry1Gb.1Ig Proteins

Amino acid position	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169
Predicted eCry1Gb.1Ig protein sequence[a]	I	V	D	S	V	E	L	L	L	M	E	E
Microbially-produced eCry1Gb.1Ig[b] protein	I	V	D	S	V	E	L	L	L	M	E	E
Purified plant-produced eCry1Gb.1Ig[b] protein	I	V	D	S	V	E	L	L	L	M	E	E

[a]Predicted sequence deduced from the nucleotide sequence.
[b]The C-terminal peptide for both the microbially-produced eCry1Gb.1Ig protein and purified plant-produced eCry1Gb.1Ig protein was identified from the chymotrypsin digest.

FIGURE 20. Amino Acid Sequence Analysis of C-Terminal Peptides of Microbially produced and Plant-produced eCry1Gb.1Ig Proteins

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(c) Equivalence of the PMI protein expressed *in planta* and a microbial system

To assess the biochemical and functional equivalence of the PMI protein produced in Event MZIR260 maize plants and PMI produced in a recombinant *Escherichia coli* expression system, the proteins from both sources were compared with respect to apparent molecular weight, immunoreactivity, peptide mass coverage, glycosylation status, and insecticidal activity (Appendix 19).

The test substance 'PMI-0120' was used in all experiments as described in Appendix 20 except the immunoreactivity and molecular weight assessment where the substance 'PMI-0114' was used instead and is described in Appendix 21.

All three study reports mentioned are provided as part of the application dossier.

Appendix 19. Ellur, Vishnutej. TK0549621. (2024). *Comparison of Phosphomannose Isomerase (PMI) Protein Expressed in Event MZIR260 Derived Maize Plants and PMI Protein Expressed in Recombinant Escherichia coli. Final Report.* Unpublished. Syngenta Crop Protection, LLC.

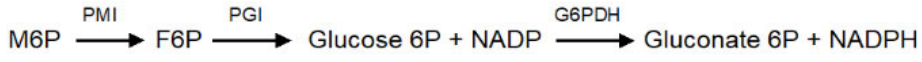
Appendix 20. Song, Feng. TK0600097. (2021). *Characterization of Microbially Produced Test Substance PMI-0120.* Unpublished. Syngenta Crop Protection, LLC.

Appendix 21. Shaw, Lauren. TK0235588. (2015). *Characterization of Microbially Produced Test Substance PMI-0114 Containing PMI Protein and Certificate of Analysis.* Unpublished.

(i) Functional activity of PMI

A validated PMI activity assay was used to determine the enzymatic activity of the microbially- and plant-produced PMI proteins (Appendix 19, Sections 3.8 & 4.5; Pages 16-17 & 22-23).

In summary, the assay monitored the production of nicotinamide adenine dinucleotide phosphate (NADPH) in coupled enzymatic reactions that included phosphoglucose isomerase (PGI) and glucose 6-phosphate dehydrogenase (G6PDH), based on the method described by Gill et al., 1986) (Gill JF, Deretic V, Chakrabarty M, 1986) and (Gracy and Noltmann, 1968) (Gracy RW, Noltmann EA, 1968) as shown in the diagram below. The production of NADPH was monitored by measuring an increase in absorbance at 340 nm.



PMI = Phosphomannose Isomerase, PGI = Phosphoglucose Isomerase, G6PDH = Glucose 6-phosophate dehydrogenase

Enzymatic activity assays included nontransgenic maize leaf crude extract as a negative control. The nontransgenic maize leaf crude extract fortified with microbially-produced PMI protein was included in the assay to determine if the plant matrix affected the PMI enzymatic activity.

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The microbially-produced PMI protein showed a mean specific activity of 331.32 U/mg PMI, and PMI protein in MZIR260 maize leaf crude extract showed a mean specific activity of 323.16 U/mg PMI, (Table 18). Nontransgenic maize leaf crude extract fortified with microbially produced PMI protein was included in the assay to determine if the plant matrix affected the PMI enzymatic activity. The mean specific activity of this sample was 303.03 U/mg PMI (Table 18), which was comparable with that of PMI protein in MZIR260 leaf crude extract and microbially produced PMI protein, thus confirming no matrix effect on the enzymatic activity and equivalent functional activity of microbially-produced PMI protein and plant-produced PMI protein from MZIR260 maize. No activity was detected from the nontransgenic maize leaf crude extract.

In Conclusion:

- Enzymatic activity assays revealed comparable specific enzymatic activities, for the microbially-produced protein and the plant-produced protein.

TABLE 18. Specific Enzymatic Activity of The Microbially-Produced and Plant Produced PMI Proteins

Sample	Assay replicate	Specific activity (U/mg PMI)	Mean specific activity (U/mg PMI)	SD (U/mg PMI)	CV (%)
Microbially-produced PMI protein	1	325.74	331.32	5.81	1.8
	2	330.90			
	3	337.33			
PMI protein in MZIR260 maize leaf crude extract	1	331.83	323.16	7.54	2.3
	2	319.55			
	3	318.10			
Nontransgenic maize leaf crude extract fortified with microbially-produced PMI protein	1	302.14	303.03	0.80	0.3
	2	303.67			
	3	303.28			
Nontransgenic maize leaf crude extract	1	ND[a]	–	–	–
	2	ND[a]			
	3	ND[a]			

[a]ND = Not detected.

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(ii) Immunoreactivity and molecular weight determination of PMI

The apparent molecular weight and immunoreactivity of both microbially- and plant produced PMI proteins were investigated using Western blot analysis and is described as part of Appendix 19, Section 3.4 & 4.2; Pages 13-14 & 17- 19.

The analysis included microbially produced PMI, purified plant-produced PMI protein, MZIR260 maize leaf crude extract, nontransgenic maize leaf crude extract fortified with microbially-produced PMI protein, and a nontransgenic maize leaf crude extract as a negative control.

In summary, samples were prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a 4-12% BoltTM Bis-Tris gel and BoltTM 2-(N-morpholino) ethanesulfonic acid (MES) running buffer. The MW standard was the Invitrogen™ SeeBlue® Plus2 pre-stained standard. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane via electroblotting. The membrane was probed with a polyclonal goat antibody capable of binding to PMI protein. Detection of PMI protein was accomplished through the binding of polyclonal donkey anti-goat antibodies conjugated with alkaline phosphatase enzyme, which catalyzed the conversion of the chromogenic substrate solution BCIP®/NBT. The blot was imaged using a Bio-Rad GS900 densitometer optical scanner. The Western blot was examined for the presence of intact immunoreactive PMI protein or other immunoreactive PMI-derived fragments.

Western blot analysis revealed immunoreactive bands corresponding to the anticipated MW of 42.8 kDa of the PMI protein (Figure 21, Lanes 2 through 5), while no immunoreactive band was observed for the nontransgenic maize leaf extract (Figure 21, Lane 6).

The Western blot analysis further identified multiple faint protein bands with molecular weights higher than the 17 kDa protein marker and lower than the intact PMI protein immunoreactive band for microbially-produced PMI protein and purified plant-produced PMI protein (Figure 21, Lanes 2 and 3). Since these protein bands cross-reacted with an PMI specific antibody and the band was not observed in a leaf extract from the nontransgenic maize leaf crude extract (Figure 21, Lane 6), they are most likely degraded fragments of the PMI protein. The absence of the faint bands lower than the intact PMI protein immunoreactive band in the MZIR260 maize leaf crude extract (Figure 21, Lane 4 and the nontransgenic maize leaf crude extract fortified with microbially-produced PMI protein Figure 21, Lane 25) is most likely due to plant matrix interference. PMI protein from MZIR260 maize leaf crude extract (Figure 21, Lane 4), revealed an immunoreactive band corresponding to the anticipated MW of ~ 42.8 kDa of the PMI protein with diminished intensity compared to the intensity of the microbially-produced PMI protein and purified plant-produced PMI protein (Figure 21, Lanes 2 and 3). A similar diminished band intensity was observed in nontransgenic maize leaf crude extract fortified with microbially produced PMI protein (Figure 21, Lane 5), indicating that the diminished intensity of the PMI protein-specific band in these samples is most likely due to plant matrix interference.

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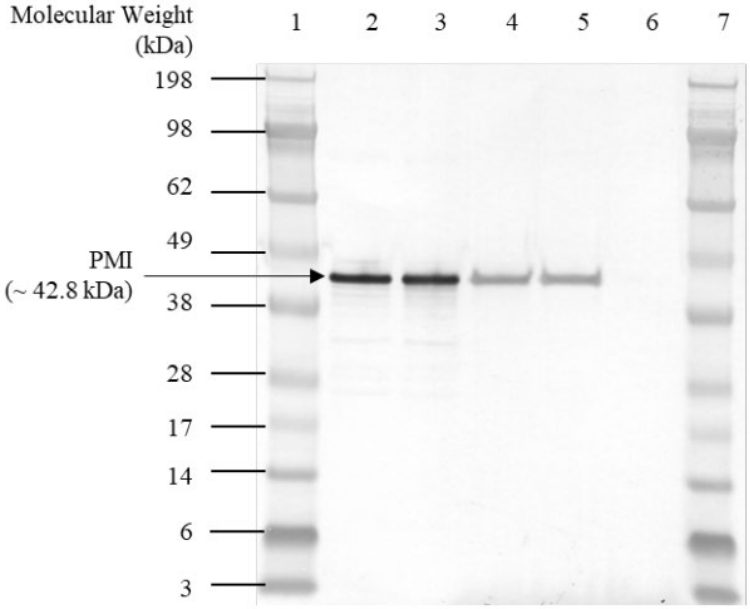
In Conclusion:

- The western blot analysis shows MW of ~ 42.8 kD of microbially-produced PMI protein and plant-produced PMI protein.
- The identity and integrity of microbially-produced PMI protein and plant-produced PMI protein are as expected.



Lane 1: Molecular weight standard
Lane 2: Microbially-produced PMI protein (3 ng PMI protein)
Lane 3: Purified plant-produced PMI protein (3 ng PMI protein)
Lane 4: MZIR260 maize leaf crude extract (3 ng PMI protein)
Lane 5: Nontransgenic maize leaf crude extract fortified with microbially-produced PMI protein (3 ng PMI protein)
Lane 6: Nontransgenic maize leaf crude extract
Lane 7: Molecular weight standard

FIGURE 21. Western Blot Analysis of the Microbially-Produced and Plant-Produced PMI Proteins

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(iii) Amino acid sequence of PMI

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

FIGURE 22. Amino acid (Deduced) Sequence of the PMI Protein

(iv) Peptide mass coverage analysis of PMI

Peptide mass coverage analysis was used to determine the identity of the microbially produced PMI protein. The study report is provided as part of this application dossier (Appendix 19, Section 3.5 – 3.6 & 4.2; Pages 14-15 & 19-20).

In summary, proteolytic peptides for peptide mass coverage analyses were produced by digesting microbially produced PMI protein and purified plant-produced PMI protein bound to ProtiFi STrap™ resin (ProtiFi LLC, Fairport, NY). Microbially-produced PMI protein was digested with 2 µg of trypsin/LysC mixture or 2 µg of chymotrypsin. Purified plant-produced PMI protein was digested with 1.2 µg of trypsin/LysC mixture or 1.2 µg of chymotrypsin. The digestions were carried out at 47°C for one hour for trypsin/LysC and 30 minutes for chymotrypsin. The samples were injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS).

```
1    MQKLINSVQN YAWGSKTALT ELYGMENPSS QPMAELWMGA  40
41   HPKSSSRVQN AAGDIVSLRD VIESDKSTLL GEAVAKRFGE  80
81   LPFLFKVLCA AQPLSIQVHP NKHNSEIGFA KENAAGIPMD 120
121  AAERNYKDPN HKPELVFALT PFLAMNAFRE FSEIVSLLQP 160
161  VAGAHPAIAH FLQQPDAERL SELFASLLNM QGEEKSRALA 200
201  ILKSALDSQQ GEPWQTIRLI SEFYPEDSGL FSPLLLNVVK 240
241  LNPGEAMFLF AETPHAYLQG VALEVMANSD NVLRAGLTPK 280
281  YIDIPELVAN VKFEAKPANQ LLTQPVKQGA ELDFPIPVDD 320
321  FAFSLHDLSD KETTISQQSA AILFCVEGDA TLWKGSQQLQ 360
361  LKPGESAFIA ANESPVTVKG HGRLARVYNK L           391
```

Underlined indicates amino acids confirmed.

FIGURE 23. Amino Acid Sequence Coverage Map for Microbially-Produced PMI Protein

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Each acquired MS/MS spectrum was analyzed using MaxQuant software, version 2.2.0.0 and 2.4.0.0 to obtain the peptide identities. The spectra of microbially produced PMI protein were searched against databases containing the PMI protein amino acid sequence and *E. coli* strain K12 reference proteome The spectra of the purified plant-produced PMI protein were searched against the databases containing PMI protein amino acid sequence and maize reference proteome (internal database). Only peptides with a false discovery rate of less than 1% were considered identified.

1	MQKLINSVQN	YAWGSKTALT	ELYGMENPSS	QPMAELWMGA	40
41	HPKSSSRVQN	AAGDIVSLRD	VIESDKSTLL	GEAVAKRFGE	80
81	LPFLFKVLCA	AQPLSIQVHP	NKHNSEIGFA	KENAAGIPMD	120
121	AAERNYKDPN	HKPELVFALT	PFLAMNAFRE	FSEIVSLLQP	160
161	VAGAHPAIAH	FLQQPDAERL	SELFASLLNM	QGEEKSRALA	200
201	ILKSALDSQQ	GEPWQTIRLI	SEFYPEDSGL	FSPLLLNVVK	240
241	LNPGEAMFLF	AETPHAYLQG	VALEVMANSD	NVLRAGLTPK	280
281	YIDIPELVAN	VKFEAKPANQ	LLTQPVKQGA	ELDFPIPVDD	320
321	FAFSLHDLSD	KETTISQQSA	AILFCVEGDA	TLWKGSQQLQ	360
361	LKPGESAFIA	ANESPVTVKG	HGRLARVY**NK**	**L**	391

Underlined indicates amino acids were confirmed; bold and not underlined indicates amino acids were not confirmed.

FIGURE 24. Amino Acid Sequence Coverage Map for Purified Plant-Produced PMI Protein

The collective analysis of the two proteolytic digests (trypsin and chymotrypsin) for the microbially-produced PMI protein resulted in coverage of 100% of the total predicted PMI protein amino acid sequence (Figure 23). The collective analysis of the two proteolytic digests (trypsin and chymotrypsin) for the purified plant-produced PMI protein resulted in coverage of 99% of the total predicted PMI protein amino acid sequence (Figure 24).

(v) N-terminal peptide sequencing of PMI

In addition to overall amino acid sequence coverage, the N-terminal peptide sequences were identified by peptide mass coverage analysis for both microbially- and purified plant produced PMI proteins and compared to the predicted PMI protein amino acid sequence (Figure 25). The N-terminal peptides of the microbially- and plant-produced PMI proteins were identical to each other and consistent with the predicted sequence (Appendix 19; Page 20).

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Amino acid position	1	2	3	4	5	6	7	8	9	10	11
Predicted PMI protein sequence[a]	M	Q	K	L	I	N	S	V	Q	N	Y
Microbially-produced PMI protein[b]	M	Q	K	L	I	N	S	V	Q	N	Y
Plant-produced PMI protein[b]	M	Q	K	L	I	N	S	V	Q	N	Y

[a]Predicted sequence deduced from the nucleotide sequence.
[b]The N-terminal peptide for both the microbially- and plant-produced PMI proteins were identified from the chymotrypsin digest.

FIGURE 25. Amino Acid Sequence Analysis of N-terminal Peptides of Microbially Produced and Purified Plant-Produced PMI Proteins

(d) Post-translational glycosylation of microbially- and plant-produced produced Cry1Gb.1Ig and PMI proteins

The microbially-produced and the purified plant produced eCry1Gb.1Ig protein (Appendix 17; Sections 3.8 & 4.4 Pages 16-17 & 22-23) and the microbially-produced and the purified plant produced PMI protein (Appendix 19; Sections 3.7 & 4.4; Pages 16 & 21-22) were analyzed with the Sigma® Glycoprotein Detection Kit for glycosylation.

In summary, the microbially-produced protein eCry1Gb.1Ig or PMI and purified plant-produced eCry1Gb.1Ig or PMI protein were analyzed with the Sigma® Glycoprotein Detection Kit for glycosylations. The eCry1Gb.1Ig protein or PMI concentration was based on ELISA quantification for purified plant-produced eCry1Gb.1Ig protein. Microbially-produced eCry1Gb.1Ig or PMI protein and purified plant-produced eCry1Gb.1Ig or PMI protein, were subjected to SDS-PAGE under reducing conditions using a 4-12% Bolt™ Bis-Tris gel and MES running buffer.

Horseradish peroxidase (HRP), a glycosylated protein, was applied to the gels at 25, 10, 5, 2.5, and 1 pmol (1100, 440, 220, 110, and 44 ng) as a positive control. Soybean trypsin inhibitor (STI), a nonglycosylated protein, was applied to the gels at 25 pmol (500 ng) as a negative control. The molecular weight standard was SeeBlue® Plus2 pre-stained standard.

Following SDS-PAGE, the proteins were electroblotted onto a PVDF membrane. While on the membrane, glycan moieties were oxidized using periodic acid, stained with Schiff's Fuchsin-Sulfite reagent, and reduced with sodium metabisulfite. Following the visualization of glycoproteins, Swift™ membrane stain was applied to the same blot to visualize the total protein on the blot and to verify the appropriate loading of the proteins. The blot was imaged using a Bio-Rad GS-900 densitometer optical scanner before and after the staining steps. Swift™ Membrane Stain was used to stain the same blot after glycosylation to visualize the total protein on the blot.

(i) Glycosylation analysis of eCry1Gb.1Ig

Figure 26 shows the results of the glycosylation analysis of the eCry1Gb.1Ig proteins. No glycosylation-stained bands were observed for the plant-produced and microbially produced eCry1Gb.1Ig proteins (Figure 26A, Lanes 8 and 9).

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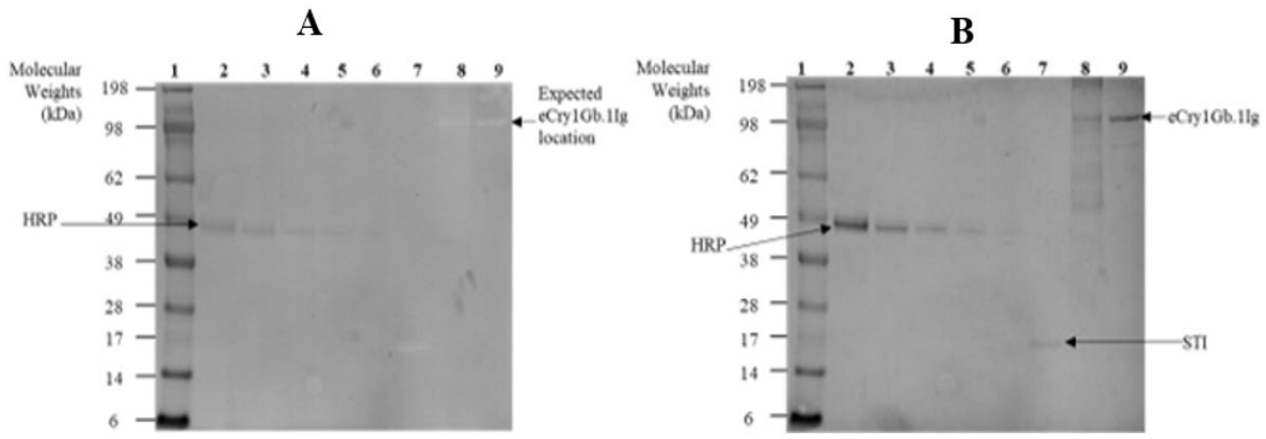
The positive control, horseradish peroxidase (HRP), generated stained bands consistently decreasing in intensity in correlation with the decreasing amounts, as expected (Figure 26A, Lanes 2 through 6). The negative control, soybean trypsin inhibitor (STI), did not generate stained bands, as expected (Figure 26A, Lane 7). These results of the positive and negative controls confirmed the suitability of the glycosylation assay to investigate the glycosylation status of both eCry1Gb.1Ig proteins.

The Swift™ Membrane Stain used to stain the same blot after glycosylation to visualize the total protein on the blot (Figure 26B) revealed the presence of two protein bands of plant-produced and microbially produced eCry1Gb.1Ig consistent with the predicted molecular weight of 133 kDa (Figure 26B, Lanes 8 and 9). In addition, protein band corresponding to STI was visualized (Figure 26B, Lane 7). These results verified the appropriate loading of eCry1Gb.1Ig proteins on the glycosylation blot.

In Conclusion:

- Glycoprotein blot analysis of microbially- and plant-produced eCry1Gb.1Ig proteins showed no evidence of post-translational glycosylation as expected.



The blot was stained for glycoprotein detection (A), followed by total protein staining (B).

Lane 1: Molecular weight standard
Lane 2: HRP positive control - 25 pmol, 1100 ng
Lane 3: HRP positive control - 10 pmol, 440 ng
Lane 4: HRP positive control - 5 pmol, 220 ng
Lane 5: HRP positive control - 2.5 pmol, 110 ng
Lane 6: HRP positive control - 1 pmol, 44 ng
Lane 7: STI negative control - 25 pmol, 500 ng
Lane 8: Purified plant-produced eCry1Gb.1Ig protein - 4.17 pmol, 555 ng
Lane 9: Microbially-produced eCry1Gb.1Ig protein - 4.17 pmol, 555 ng

FIGURE 26. Glycosylation Analysis of the Microbially-Produced and Plant-Produced eCry1Gb.1Ig

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(ii) Glycosylation analysis of PMI

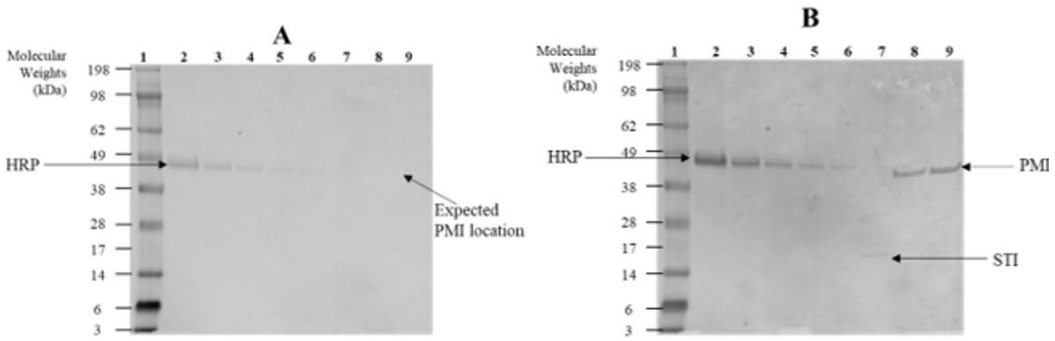
Figure 27 A shows the results of the glycosylation analysis of the PMI protein. No glycosylation-stained bands were observed for the microbially-produced and plant-produced PMI proteins (Figure 27A, Lanes 8 and 9).

The positive control, HRP, generated stained bands consistently decreasing in intensity in correlation with the decreasing amounts, as expected (Figure 27A, Lanes 2 through 6). The negative control, STI, did not generate stained bands, as expected (Figure 27A, Lane 7). These results of the positive and negative controls confirmed the suitability of the glycosylation assay to investigate the glycosylation status of both PMI proteins.

The Swift™ membrane stain used to stain the same blot after glycosylation analysis was done, to visualize the total protein on the blot (Figure 27B), revealed the presence of two protein bands of microbially-produced and plant-produced PMI proteins consistent with the predicted molecular weight of 42.8 kDa (Figure 27B, Lanes 8 and 9). In addition, the protein band corresponding to STI was also visualized (Figures 27B, Lane 7). These results verified the appropriate loading of PMI proteins on the glycosylation blot.

In Conclusion:

- Glycoprotein blot analysis of microbially- and plant-produced PMI proteins showed no evidence of post-translational glycosylation as expected.



The blot was stained for glycoprotein detection (A), followed by total protein staining (B).

Lane 1: Molecular weight standard
Lane 2: HRP positive control - 25 pmol, 1100 ng
Lane 3: HRP positive control - 10 pmol, 440 ng
Lane 4: HRP positive control - 5 pmol, 220 ng
Lane 5: HRP positive control - 2.5 pmol, 110 ng
Lane 6: HRP positive control - 1 pmol, 44 ng
Lane 7: STI negative control - 25 pmol, 500 ng
Lane 8: PMI protein purified from MZIR260 maize leaf extract - 14.72 pmol, 630 ng
Lane 9: Microbially-produced PMI protein - 14.72 pmol, 630 ngC.

FIGURE 27. Glycosylation Analysis of the Microbially-Produced and Plant-Produced PMI

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(e) Where any ORFs have been identified, bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs.

Refer to Section 2.03 c (iv).

(f) Conclusion - Characterisation of the NEPs

A profile of eCry1Gb.1Ig and PMI protein concentrations present in MZIR260 maize in various tissue types and developmental stages across four different field environments was assembled.

The biochemical and functional equivalence of the eCry1Gb.1Ig protein produced in MZIR260 maize plants and those produced in a recombinant *E. coli* expression system were confirmed by apparent molecular weight, immunoreactivity, peptide mass coverage, glycosylation status, and insecticidal activity (Ellur and Wu, 2024 *unpublished*). The eCry1Gb.1Ig protein from both sources was demonstrated to have the same molecular weight of approximately 133 kilodaltons, cross-reacted with the same eCry1Gb.1Ig-specific antibody, and comparable 50% lethal concentration (LC_{50}) values towards soybean looper (SBL, *Chrysodeixis includens*). Peptide mass coverage analysis of the microbially- and the plant-produced eCry1Gb.1Ig protein resulted in 96% and 88% coverage of the predicted eCry1Gb.1Ig protein amino acid sequence, respectively. In addition, the N-terminal, and C-terminal sequences for both microbially- and plant-produced eCry1Gb.1Ig proteins were identical and consistent with the predicted protein amino acid sequence. Glycoprotein blot analysis of microbially- and plant-produced eCry1Gb.1Ig proteins showed no evidence of post-translational glycosylation.

The biochemical and functional equivalence of both the plant-produced and microbially-produced PMI proteins were confirmed by apparent molecular weight, immunoreactivity, and enzymatic activity (Ellur, 2024 *unpublished*). PMI proteins from both sources were demonstrated to have the same molecular weight of 42.8 kilodaltons, reacted with the same PMI-specific antibody, and comparable specific enzymatic activities, with 303.03 U/mg for the microbially-produced PMI protein in the presence of nontransgenic leaf extract, and 323.16 U/mg PMI protein for the plant-produced protein. Peptide mass coverage analysis of the microbially- and the plant-produced PMI proteins resulted in 100% and 99% coverage of the predicted PMI protein amino acid sequence, respectively. In addition, the N-terminal sequence for both microbially- and plant-produced PMI proteins were identical and consistent with the predicted protein amino acid sequence. Glycoprotein blot analysis of microbially- and plant-produced PMI proteins showed no evidence of post-translational glycosylation.

The data collectively show that the microbially-produced eCry1Gb.1Ig protein or the microbially-produced PMI protein are functionally and biochemically equivalent to the plant-produced versions with respect to apparent molecular weight, immunoreactivity, peptide mass coverage, glycosylation status, and enzymatic activity. Microbially produced versions of either protein are suitable surrogates to evaluate the safety of these proteins *in planta* - as MZIR260.

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Section 3.02 Safety Assessment of Introduced Proteins Where the Protein is Identical to a Protein Previously Assessed by FSANZ – PMI

(a) Information on the potential toxicity and allergenicity of PMI

PMI catalyzes the interconversion of mannose 6-phosphate and fructose 6-phosphate and has utility as a selectable marker for the transformation of many plant species (Bojsen, 1998) and (Negrotto *et al*., 2000). Plant cells that have been transformed with the PMI protein are able to survive and grow on media containing mannose as a primary carbon source. Under the same conditions, plant cells lacking PMI accumulate mannose-6-phosphate and fail to grow.

The PMI protein sequence expressed in MZIR260 corn is identical to several Syngenta applications previously assessed by FSANZ (Table 19). In addition, applications from other companies have been assessed by FSANZ including DP23211 maize (A1202, FSANZ 2020) and DP51291 (A1270, FSANZ 2023). None of these assessments have raised any general safety concerns or evidence of adverse health effects in humans. Therefore, the safety data presented in this application includes bioinformatics search using 2023 databases to determine if the PMI amino acid sequence has significant sequence similarity with proteins that are known or putative toxins or allergens of mammalian concern.

Company	Application	Event	Reference
Syngenta	A1060	Event 5307 maize	FSANZ, 2012
Syngenta	A1001	Event MIR162 maize	FSANZ, 2008a
Syngenta	A580	Event 3272 maize	FSANZ, 2008b

TABLE 19. Syngenta Maize Applications Approved by FSANZ Containing the Identical PMI protein as found in MZIR260

(i) Source organism

The source organism of PMI is *E. coli* K-12.

(ii) History of safe use (HOSU) in food

PMI is widely present in nature and is likely that small amounts of PMI proteins from various sources have always been present in the food and feed supply due to its ubiquitous occurrence. The *pmi* gene from *E. coli* K-12 has been used as a selective marker in numerous commercialized transgenic crops. The food and feed safety of genetically modified crops expressing the *pmi* gene has been extensively evaluated by regulatory agencies worldwide, which also demonstrates the safety of *E. coli* K-12 being used as a source organism.

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(iii) Bioinformatic comparisons of the amino acid sequence with potential toxins

Bioinformatic analysis determined if the PMI amino acid sequence has significant sequence similarity with proteins that are known or putative toxins of mammalian concern (Appendix 22). The study report is provided as part of this application dossier.

Appendix 22. Joshi, Saurabh. RIR-0002627-23. (2023). *Phosphomannose Isomerase (PMI) Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins. Assessment.* Unpublished. Syngenta Crop Protection, LLC.

In summary, the PMI sequence of 391 amino acids (Figure 22) was systematically compared with two databases using the Basic Local Alignment Search Tool for Proteins (BLASTP, version 2.8.1+) program (Altschul et al., 1997) to determine whether it has significant similarity to known or putative mammalian toxins.

The compiled library of all protein sequences at the National Center for Biotechnology Information (NCBI) Entrez® Protein Database (NCBI, 2023c) was first searched to identify relevant proteins with significant sequence similarity to PMI. Proteins meeting the similarity inclusion criteria were further evaluated to determine whether PMI possesses the potential for toxicity in mammals. A curated database of known or putative mammalian toxin sequences in the Syngenta toxin database (version, 2023) was used in alignment comparisons using BLASTP to more clearly identify all known mammalian toxins that share significant similarity with PMI.

For each BLASTP alignment, sequence similarity is reported using an *E*-value, which is a measure of the probability that potentially significant alignments between sequences occurred by chance. Search results involving comparisons between proteins with highly similar sequences yield *E*-values approaching zero; the probability that sequence similarities occurred by random chance, and not due to their inherent taxonomic or functional relatedness, increases with higher *E*-values (Ponting, 2001).

Alignments requiring further evaluation were identified using an *E*-value threshold of 1×10^{-5}. This procedure was used to identify proteins that show significant sequence similarity to the PMI amino acid sequence and determine if PMI shares biologically relevant sequence similarity with known or putative mammalian protein toxins.

NCBI Database Alignments:

The NCBI Entrez® Protein Database search identified the top 1000 sequences with the most significant similarity to the PMI amino acid sequence (i.e., *E*-values less than 1×10^{-5}) and none showed sequence similarity to known or putative mammalian protein toxins. All the 1000 sequences from 19 species were identified as PMI proteins.

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Syngenta Toxin Database Alignments:

There were no proteins in the Syngenta toxin database with significant sequence similarity (*E*-value < 1×10^{-5}) to the PMI protein. Six alignments below the upper reportable *E*-value (*E*-value < 10) were observed. The most similar alignment was with a protein from the database which had an *E*-value of 2.3998. This *E-value* is greater than the significance threshold *E*-value of 1×10^{-5}, indicating that none of these alignments is of biological significance in terms of potential toxicity.

When the screening threshold *E*-value is set at 1×10^{-5} or lower, PMI protein showed no sequence similarity to any known or putative mammalian protein toxins in either NCBI Entrez® database or Syngenta toxin database. Results from both database comparisons confirm that PMI is not a mammalian toxin, nor does PMI share significant sequence similarity with other known or putative mammalian protein toxins.

In Conclusion:

- Bioinformatic searches of the PMI sequence using 2023 databases identified no biologically relevant amino acid sequence similarity to any known or putative mammalian toxins

(iv) Bioinformatic comparisons of the amino acid sequence with potential allergens

Bioinformatic analysis determined whether the PMI amino acid sequence has biologically relevant similarity to amino acid sequences of known or putative allergens. (Appendix 23). The study report is provided as part of this application dossier.

Appendix 23. Joshi, Saurabh. RIR-0001201-23. (2023). *Phosphomannose Isomerase (PMI) Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens.* Unpublished. Syngenta Crop Protection, LLC.

In summary, to determine whether the PMI protein has biologically relevant similarity to known or putative allergens, its amino acid sequence was compared to the 2631 amino acid sequences of known or putative allergens documented in the curated Comprehensive Protein Allergen Resource (COMPARE) database, version 2023.

The comparison was conducted in two different search strategies, a full-length sequence search using FASTA (FAST-All sequence alignment software package), and a separate search for exact matches of eight contiguous amino acids. Such alignments are considered to indicate the potential for immunologically relevant sequence similarity (Codex, 2009).

In the FASTA search, there were 31 alignments with *E*-values less than 10, none of which exceeded the minimum significance criteria of greater than 35.0 % shared identity over a minimum of 80 amino acids of alignment length. Therefore, no sequence similarity greater than 35% shared

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identity over 80 or more amino acids was observed between the PMI amino acid sequence and any entry in the COMPARE database, (version 2023).

In the eight amino acid match searches, a single match between PMI and a known allergen, the frog α-parvalbumin from *Rana* species CH2001, (Accession Number CAC83047.1) was observed. Further investigation was conducted using serum IgE from a patient allergic to the frog α-parvalbumin from *Rana* species CH2001. The serum screening analysis demonstrated reactivity towards the α-parvalbumin but no IgE-reactivity with PMI. These results support the conclusion that the shared eight amino acid sequence in PMI was not evidence of potential cross-reactivity between α-parvalbumin and PMI. This information is not new has been provided in previous safety assessments approved by FSANZ.

In Conclusion:

- Bioinformatic searches of the PMI sequence using the 2023 COMPARE allergen database identified no biologically relevant amino acid sequence similarity to any known or putative protein allergens

(b) Conclusion on PMI safety

The weight-of-evidence presented supports the conclusion that PMI does not exhibit characteristics typically associated with food toxins or allergens. Specifically, the PMI protein does not share biologically relevant amino acid sequence similarity to known or putative known toxins or allergens. Up-to-date bioinformatics analysis aligns with the conclusions presented in previous safety assessments, that also mention that the PMI protein does not share similarity to any known biological relevant toxins or allergens of mammalian concern.

These findings, coupled with the documented history of safe use of the gene's source organism, the established Mode of Action and safety record of PMI proteins indicate that PMI protein is unlikely to be toxic or allergenic to human and animal consumers. Consequently, no negative impacts on animal or human health are anticipated from the intake of the PMI protein.

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Section 3.03 Safety Assessment (Full) of Introduced Proteins Where the Protein is NOT Identical to a Protein Previously Assessed by FSANZ - eCry1Gb.1Ig

(a) Information on the potential toxicity and allergenicity of eCry1Gb.1Ig

To date, the eCry1Gb.1Ig protein has not been released in a GM food crop. To evaluate the safety of the eCry1Gb.1Ig expressed in the Event MZIR260 maize, a weight-of-evidence approach was taken based on several key types of data, including information about the source organism from which the *eCry1Gb.1Ig* gene originates; history of safe use; a bioinformatic assessment of whether the introduced protein shares significant amino acid sequence similarity to known or putative toxic or allergenic proteins and susceptibility of the protein to heat and simulated human digestion fluids. This method aligns with the recommendations by the Codex Alimentarius Commission a useful predictor of toxicity and allergenicity when consumed by humans or animals in food (Codex, 2009).

(i) Source of the protein

The source organism of eCry1Gb.1Ig is *Bacillus thuringiensis (B. thuringiensis*).

(ii) History of safe use (HOSU) in food

To-date, the eCry1Gb.1Ig protein has not been released in a GM food crop however, maize crops planted in North America are predominately genetically modified with a range of *Cry* genes from *Bacillus thuringiensi*s including Cry1Gb and Cry1Ig that was used to engineer eCry1Gb.1Ig.

Furthermore, while eCry1Gb.1Ig shows a unique mode of action against FAW, it remains structurally similar with, and has similar biological function to other Cry proteins. Specifically, the eCry1Gb.1Ig protein has been demonstrated to share a general mode-of-action (MoA) with known commercial Cry toxins. Many of which have a long history of safe use (Zwack et al., 2024).

The ingested protoxin can be proteolytically activated by the contents of insect gastric fluid and subsequently binds to membrane of midgut epithelial cells, forms pores, and leads to cell death. This series of events is consistent with current models of Cry protein toxicity. However, the studies also revealed that the target site of eCry1Gb.1Ig does not overlap with those of other *Bt* toxins currently used against FAW.

(iii) A bioinformatic comparison of the amino acid sequence of eCry1Gb.1Ig to known protein toxins

A study was completed to determine if the eCry1Gb.1Ig amino acid sequence has significant sequence similarity with proteins that are known or putative toxins of mammalian concern (Appendix 24). The study report is provided as part of this application dossier.

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Appendix 24. Joshi, Saurabh. RIR-0006809-23. (2023). *eCry1Gb.1Ig Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins. Assessment.* Unpublished. Syngenta Seeds, LLC.

In summary, the eCry1Gb.1Ig amino acid sequence (Figure 16) was systematically compared with two databases using the Basic Local Alignment Search Tool for Proteins (BLASTP, version 2.8.1+) program (Altschul *et al.* 1997) to determine whether it has significant similarity to known or putative mammalian toxins.

The compiled library of all protein sequences at the National Center for Biotechnology Information (NCBI) Entrez® Protein Database (2023) was first searched to identify relevant proteins with significant sequence similarity to eCry1Gb.1Ig. Proteins meeting the similarity inclusion criteria were further evaluated to determine whether eCry1Gb.1Ig possesses the potential for toxicity in mammals. A curated database of known or putative mammalian toxin sequences - Syngenta toxin database (version 2023) was used in alignment comparisons using BLASTP to more clearly identify all known mammalian toxins that share significant similarity with eCry1Gb.1Ig.

For each BLASTP alignment, sequence similarity is reported using an *E*-value, which is a measure of the probability that potentially significant alignments between sequences occurred by chance. Search results involving comparisons between proteins with highly similar sequences yield *E*-values approaching zero; the probability that sequence similarities occurred by random chance, and not due to their inherent taxonomic or functional relatedness, increases with higher *E*-values (Ponting 2001).

NCBI Database Alignments:

The NCBI Entrez® Protein Database search identified the top 1000 sequences with the most significant similarity to the eCry1Gb.1Ig amino acid sequence (i.e., *E-values* less than 1×10^{-5}) none of which showed sequence similarity to known or putative mammalian protein toxins. Of the 1000 sequences, 950 proteins from 22 species were identified as "Cry" proteins. An additional 47 proteins from 6 species were identified as hypothetical / unknown proteins. The *E-values* for alignments between these sequences and the eCry1Gb.1Ig amino acid sequence ranged from 0 to 3.90771×10^{-131}. An additional 3 proteins from 1 species were identified as vector proteins. The *E-value* for alignments between these sequences and the eCry1Gb.1Ig amino acid sequence was 0.

Syngenta Toxin Database Alignments:

There were no proteins in the Syngenta toxin database with significant sequence similarity (*E-value* $< 1 \times 10^{-5}$) to the eCry1Gb.1Ig protein. Nine alignments below the upper reportable *E-value* (*E-value* < 10) were observed. The most similar alignment was with a protein from the database which had an *E-value* of 1.11954. This *E-value* is greater than the significance threshold *E-value* of 1×10^{-5}, indicating that none of these alignments is of biological significance in terms of potential toxicity.

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When the screening threshold *E*-value is set at 1×10^{-5} or lower, the eCry1Gb.1Ig protein showed no sequence similarity to any known or putative mammalian protein toxins in either the NCBI Entrez® database or the Syngenta toxin database. Results from both database comparisons confirm that eCry1Gb.1Ig does not share significant sequence similarity with any known or putative mammalian protein toxins.

In Conclusion:

- eCry1Gb.1Ig amino acid sequence shows no biologically relevant sequence similarity to any known or putative mammalian toxins

(iv) A bioinformatic comparison of the amino acid sequence of eCry1Gb.1Ig to known protein allergens

Bioinformatic analysis determined whether the eCry1Gb.1Ig amino acid sequence has biologically relevant similarity to amino acid sequences of known or putative allergens (Appendix 25). The study report is provided as part of this application dossier.

Appendix 25. Joshi, Saurabh. RIR-0006807-23. (2023). *eCry1Gb.1g Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens. Assessment.* Unpublished. Syngenta Crop Protection, LLC.

In summary, to determine whether the eCry1Gb.1Ig protein has biologically relevant similarity to known or putative allergens, its amino acid sequence was compared to the 2631 amino acid sequences of known or putative allergens documented in the curated Comprehensive Protein Allergen Resource (COMPARE) database, version 2023.

The comparison was conducted in two different search strategies, a full-length sequence search using FASTA (FAST-All sequence alignment software package), and a separate search for exact matches of eight contiguous amino acids. Such alignments are considered to indicate the potential for immunologically relevant sequence similarity (Codex, 2009).

The FASTA search returned 43 alignments with *E-values* less than 10, none of which exceeded the minimum significance criteria of 35.0% shared identity over a minimum of 80 amino acids of alignment length. In addition, no matches between any sequence of eight contiguous amino acids of eCry1Gb.1Ig and any entry in the COMPARE database. were found. Therefore, no significant sequence similarity was observed between the eCry1Gb.1Ig amino acid sequence and any entry in the COMPARE database (version 2023).

In Conclusion:

- Cry1Gb.1Ig amino acid sequence shows no biologically relevant sequence similarity to any known or putative protein allergens.

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(v) Structural stability of the protein to enzymes found in gastrointestinal environments, heat and/or acid

a) In Vitro Digestibility of Microbially Produced ECRY1GB.1IG Protein under Simulated Mammalian Gastric Conditions

A study was conducted to assess the *in vitro* digestibility of microbially produced eCry1Gb.1Ig protein in simulated mammalian gastric fluid (SGF) over a 60-minute time course at 37°C using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Western blot analyses (Appendix 26). The study report is provided as part of this application dossier.

Appendix 26. Perry, Alexandra. TK0549448. (2024). *In Vitro Digestibility of Microbially Produced eCry1Gb.1Ig Protein under Simulated Mammalian Gastric Conditions. Final Report.* Unpublished. Eurofins Agrosciences Services.

In summary, the SGF digestibility assay for eCry1Gb.1Ig was performed at 37°C ± 2°C over a 60-minute time course with samples taken at 0, 1, 2, 5, 10, 30, and 60 minutes. The SDS-PAGE gel was stained with Coomassie® blue which allowed visualization of all the proteins and fragments present in the digestion mixture. The gel was examined for the presence of bands consistent with the molecular weight of intact eCry1Gb.1Ig protein (approximately 133 kDa), bands corresponding to eCry1Gb.1Ig-derived fragments, and bands corresponding to pepsin (approximately 34.6 kDa). For Western blotting, proteins were transferred from the SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane via electroblotting, and the membrane was probed with a polyclonal goat antibody capable of detecting eCry1Gb.1Ig protein. Detection of eCry1Gb.1Ig protein was accomplished through binding of secondary polyclonal donkey anti-goat antibodies conjugated with alkaline phosphatase enzyme, which catalyzed the conversion of the 5- bromo-4-chloro-3-indolyl phosphate (BCIP®)/ nitro blue tetrazolium (NBT) chromogenic substrate solution. The Western blot was visually examined for the presence of intact immunoreactive eCry1Gb.1Ig protein (approximately 133 kDa) or other immunoreactive eCry1Gb.1Ig-derived fragments.

The SDS-PAGE analysis (Figure 28) results suggest that the eCry1Gb.1Ig protein is readily digested in SGF in one minute. The band corresponding to the full length eCry1Gb.1Ig at time zero (Figure 28, Lane 7) was no longer visible after one minute incubation in SGF (Figure 28, Lane 8). Two bands around 3 and 4 kDa appeared after one minute of eCry1Gb.1Ig digestion and were present throughout the 60 minutes time course (Figure 28, Lanes 8 through 13). The approximate molecular weight of these two bands present in Figure 28, Lanes 8 through 13 was estimated to be approximately 3 and 4 kDa, by analysing the migration of these fragments relative to the migration of the molecular weight marker on the SDS-PAGE gel. The protein band present in the SGF control (Figure 28, Lanes 2 and 3), at approximately 34.6 kDa, corresponded to the molecular weight of pepsin. The approximately 34.6 kDa pepsin band was also visible in the in vitro digestibility assay samples (Figure 28, Lanes 7 through 13). There was no significant degradation of pepsin observed in the SGF control (Lane 3) when compared to the time zero sample (Figure 28, Lane 2).

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The eCry1Gb.1Ig protein incubated in SGF without pepsin (eCry1Gb.1Ig control) showed no significant degradation over the 60 minutes (Figure 28, Lane 5), which indicates that the hydrolysis of eCry1Gb.1Ig protein, seen in the SGF samples (Figure 28, Lanes 8 through 13), can be attributed to pepsin. Similar band intensities were visualized between the time zero *in vitro* digestibility assay sample (Figure 28, Lane 7) and the eCry1Gb.1Ig time zero control (Figure 28, Lane 4), which met the loading acceptance criterion and confirmed that equal amounts of eCry1Gb.1Ig protein were applied to the SDS-PAGE.

On the SDS-PAGE gel, four of five LOD samples generated visible eCry1Gb.1Ig bands and one LOD sample did not generate any visible eCry1Gb.1Ig bands, thus meeting the acceptance criteria. The lowest amount of eCry1Gb.1Ig visible on the gel (Figure 27, Lanes 15 through 19) was 1.95 ng (Figure 28, Lane 18). Therefore, the LOD of eCry1Gb.1Ig for the SDS-PAGE used in this study was determined to be 1.95 ng.

The Western blot analysis (Figure 29) results confirm that eCry1Gb.1Ig protein is readily digested in SGF. After incubation of eCry1Gb.1Ig protein in SGF with pepsin for one minute (Figure 29, Lane 8), no protein bands representing either intact eCry1Gb.1Ig, eCry1Gb.1Ig oligomers, or eCry1Gb.1Ig -derived fragments were detected.

The eCry1Gb.1Ig protein incubated in SGF buffer without pepsin (eCry1Gb.1Ig control) showed no significant degradation over the 60 minutes (Figure 29, Lane 5), which met the acceptance criterion and indicated that the hydrolysis of eCry1Gb.1Ig observed for the SGF samples (Figure 29, Lanes 8 through 13) can be attributed to pepsin.

Similar band intensities were visualized between the time zero *in vitro* digestibility assay sample (Figure 29, Lane 7) and the eCry1Gb.1Ig control at time zero (Figure 29, Lane 4), which met the acceptance criterion and confirmed that equal amounts of eCry1Gb.1Ig were applied to the SDS-PAGE and electroblotted.

On the blot, four of five LOD samples generated visible eCry1Gb.1Ig bands and one LOD sample did not generate any visible eCry1Gb.1Ig bands, thus meeting the acceptance criteria. The lowest amount of eCry1Gb.1Ig visible on the blot (Figure 29, Lanes 15 through 19) was 1.25 ng (Figure 28, Lane 18). Therefore, the LOD of eCry1Gb.1Ig for the Western blot used in this study was determined to be 1.25 ng.

In Conclusion:

- eCry1Gb.1Ig protein is readily digested by the mammalian gastric enzyme pepsin.

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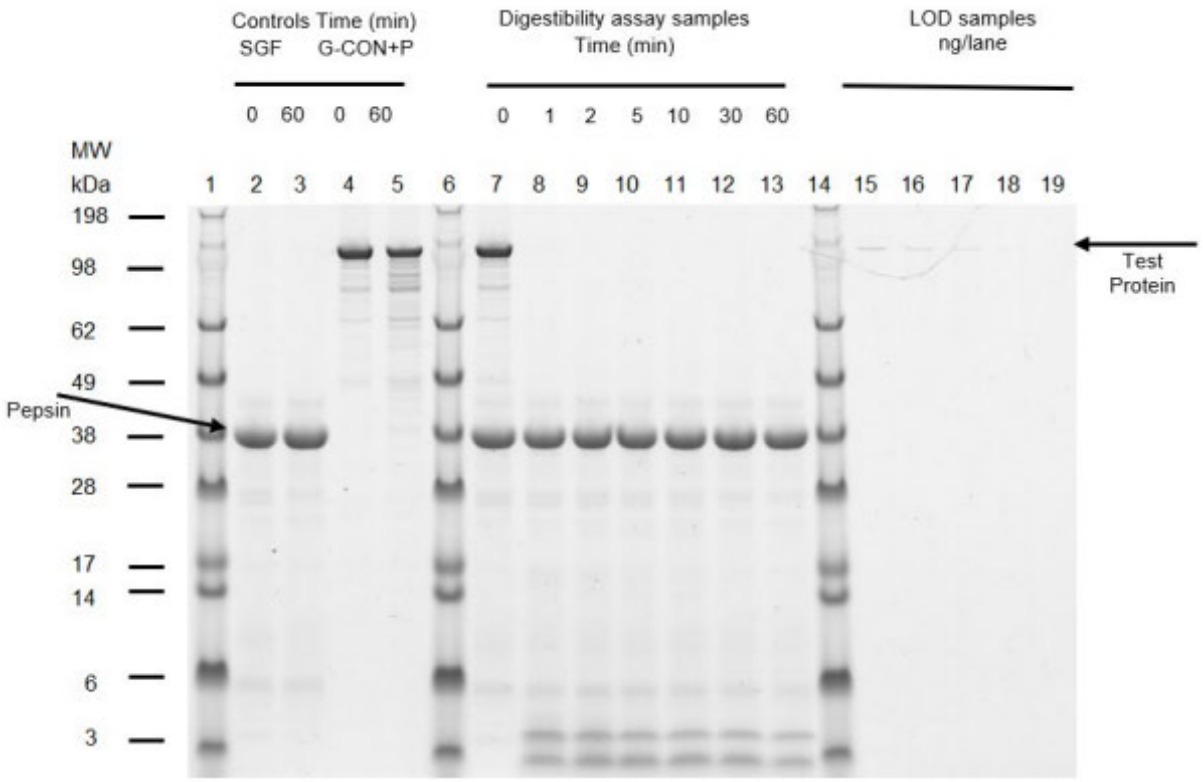

Lane 1: Molecular weight standard
Lane 2: SGF control- time zero
Lane 3: SGF control- 60 minutes
Lane 4: eCry1Gb.1Ig control (eCry1Gb.1Ig in SGF without pepsin) – time zero
Lane 5: eCry1Gb.1Ig control (eCry1Gb.1Ig in SGF without pepsin) – 60 minutes
Lane 6: Molecular weight standard
Lane 7: *in vitro* digestibility assay - time zero
Lane 8: *in vitro* digestibility assay - 1 minute
Lane 9: *in vitro* digestibility assay - 2 minutes
Lane 10: *in vitro* digestibility assay - 5 minutes
Lane 11: *in vitro* digestibility assay - 10 minutes
Lane 12: *in vitro* digestibility assay - 30 minutes
Lane 13: *in vitro* digestibility assay - 60 minutes
Lane 14: Molecular weight standard
Lane 15: 15.63 ng eCry1Gb.1Ig for LOD determination
Lane 16: 7.81 ng eCry1Gb.1Ig for LOD determination
Lane 17: 3.91 ng eCry1Gb.1Ig for LOD determination
Lane 18 1.95 ng eCry1Gb.1Ig for LOD determination
Lane 19: 0.98 ng eCry1Gb.1Ig for LOD determination

The molecular weight of pepsin is approximately 34.6 kDa. The predicted molecular weight of eCry1Gb.1Ig is approximately 133 kDa. The G-Con and the time course samples were loaded at 0.75 µg of eCry1Gb.1Ig per well.

FIGURE 28. SDS-PAGE Analysis of eCry1Gb.1Ig Following Digestion in SGF

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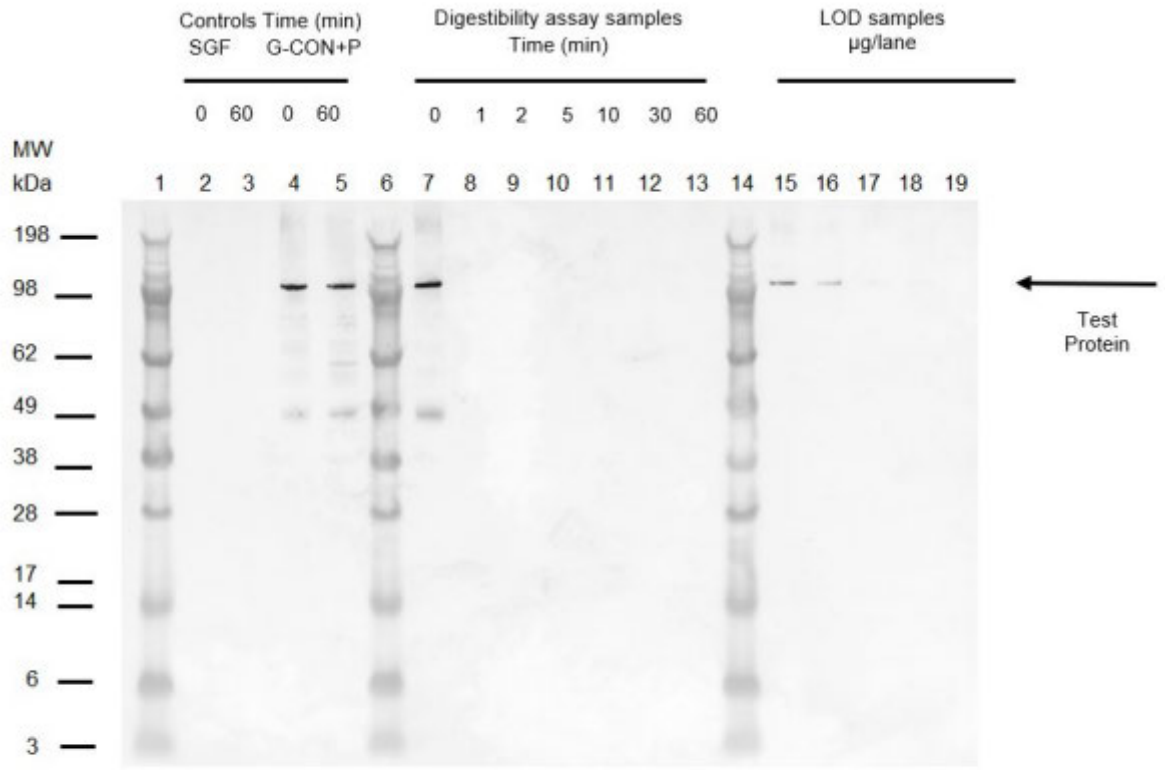

Lane 1: Molecular weight standard
Lane 2: SGF control – time zero
Lane 3: SGF control – 60 minutes
Lane 4: eCry1Gb.1Ig control (eCry1Gb.1Ig in SGF without pepsin) – time zero
Lane 5: eCry1Gb.1Ig control (eCry1Gb.1Ig in SGF without pepsin) – 60 minutes
Lane 6: Molecular weight standard
Lane 7: *in vitro* digestibility assay - time zero
Lane 8: *in vitro* digestibility assay - 1 minute
Lane 9: *in vitro* digestibility assay - 2 minutes
Lane 10: *in vitro* digestibility assay - 5 minutes
Lane 11: *in vitro* digestibility assay - 10 minutes
Lane 12: *in vitro* digestibility assay - 30 minutes
Lane 13: *in vitro* digestibility assay - 60 minutes
Lane 14: Molecular weight standard
Lane 15: 10 ng eCry1Gb.1Ig for LOD determination
Lane 16: 5 ng eCry1Gb.1Ig for LOD determination
Lane 17: 2.5 ng eCry1Gb.1Ig for LOD determination
Lane 18: 1.25 ng eCry1Gb.1Ig for LOD determination
Lane 19: 0.63 ng eCry1Gb.1Ig for LOD determination

The predicted molecular weight of eCry1Gb.1Ig is approximately 133 kDa. The G-Con and the time course samples were loaded at 50 ng of eCry1Gb.1Ig per well.

FIGURE 29. Western Blot Analysis of eCry1Gb.1Ig Following Digestion in SGF

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b) Effect of Temperature on the Bioactivity of eCry1Gb.1Ig Protein

A study was conducted to investigate the effect of temperature on the insecticidal activity of eCry1Gb.1Ig protein (Appendix 27). The study report is provided as part of this application dossier.

Appendix 27. Wu, Jianhong. TK0549452. (2024). *Effect of Temperature on the Bioactivity of eCry1Gb.1Ig Protein.* Unpublished. Syngenta Crop Protection, LLC.

In summary, eCry1Gb.1Ig protein in test substance ECRY1GB.1IG-0121 was incubated at 4°C (baseline control), 25°C, 37°C, 65°C, and 95°C for 30 minutes and the loss of bioactivity, as compared to the baseline control sample, was determined by bioassay against FAW larvae.

The FAW bioassay results (Table 20) showed that the eCry1Gb.1Ig protein samples at a dose of 497 ng eCry1Gb.1Ig /cm2 diet surface had a baseline insecticidal activity as shown by the FAW mortality of 85.4% (95% confidence interval (CI) of 82.7% – 88.1%). After incubation at 25°C for 30 minutes, the eCry1Gb.1Ig samples retained a similar activity with a mortality of 81.9% (95% CI of 79.3% – 84.6%). However, when incubated at elevated temperatures of 37°C or 65°C for 30 minutes, the eCry1Gb.1Ig samples had significantly reduced activities, with respective FAW mortalities of 50.7% (95% CI of 48.0% – 53.4%) and 30.1% (95% CI of 27.4% – 32.8%). After incubation at 95°C for 30 minutes, the eCry1Gb.1Ig samples had a mortality of 1.4% (95% CI of -1.3% – 4.1%), which was similar to the negative control.

In Conclusion:

- eCry1Gb.1Ig protein becomes denatured, and therefore inactivated, after heat treatment.

TABLE 20. Comparison of Insecticidal Activities (120 hours) of the eCry1Gb.1Ig Samples After Temperature Treatments (25°C, 37°C, 65°C, and 95°C) with that of the Baseline Control Samples (4°C)

Temperature treatment	Test #	Dead #	Mortality%	95% CI (%)	*P*[a]
4°C	144	123	85.4	82.7 – 88.1	-
25°C	144	118	81.9	79.3 – 84.6	0.0829
37°C	144	73	50.7	48.0 – 53.4	**<0.0001**
65°C	143	43	30.1	27.4 – 32.8	**<0.0001**
95°C	144	2	1.4	-1.3 – 4.1	**<0.0001**
Negative control	72	0	0	-	-

[a]bold values indicate significant difference based on an alpha level of 0.05.

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(vi) Specific serum screening where the protein is derived from a source known to be allergenic or has sequence homology with a known allergen

Not applicable

(vii) Role of the protein, if any, in the elicitation of gluten-sensitive enteropathy, in cases where the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains.

Not applicable

(b) Conclusions on potential toxicity and allergenicity of the eCry1Gb.1Ig protein

The weight-of-evidence presented supports the conclusion that eCry1Gb.1Ig does not exhibit characteristics typically associated with food toxins or allergens. The full length eCry1Gb.1Ig protein is sensitive to elevated temperatures that are commonly used during food processing and was shown to be readily digested under typical mammalian digestive conditions. These findings suggest a minimal likelihood that the intact eCry1Gb.1Ig protein could withstand food processing and digestion and be absorbed through the gastrointestinal system. Furthermore, the eCry1Gb.1Ig amino acid sequence does not share significant sequence similarity to known protein toxins or allergens as determined by up-to-date bioinformatic searches.

These findings, coupled with the documented history of safe use of the gene's source organism, the established Mode of Action and safety record of Cry proteins indicate that eCry1Gb.1Ig protein is unlikely to be toxic or allergenic to human and animal consumers. Consequently, no negative impacts on animal or human health are anticipated from the intake of the eCry1Gb.1Ig protein.

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Section 3.04 Novel Herbicide Metabolites in GM Herbicide - Tolerant Plants

Not applicable.

Section 3.05 Compositional Analyses

Levels of key food and feed nutrients and antinutrients in forage and grain from Event MZIR260 maize were compared to the levels in nontransgenic, near-isogenic control maize as part of a food and feed nutritional assessment. Comparisons to reference and/or literature ranges were made to determine the range of natural variation and to establish the *biological significance* of any identified statistical differences. Through this process it was determined whether any statistically significant differences required further investigation. (Appendix 28).

The study report is provided in this application dossier.

Appendix 28. Makani, Mildred. RIR-0007247. (2024). *Compositional Analysis of Forage and Grain from Event MZIR260 Maize Grown in the USA in 2022. Report.* Unpublished. Syngenta Seeds, LLC.

In summary, Event MZIR260 maize (test), the corresponding nontransgenic, near-isogenic maize (control), and six nontransgenic maize reference hybrids were grown at eleven locations in the United States during the 2022 growing season.

The map of the trial locations is provided in Figure 30.

Three of the six reference hybrids were grown at each location, and all entries were grown in a randomized complete block design with four replicates per entry. Forage and grain samples were collected and analyzed for key food and feed nutrients and anti-nutrients.

The components in maize forage and grain were analysed according to OECD guidelines (Table 21). Forage was analyzed for proximates, fiber, and minerals; grain was analyzed for proximates, fiber, starch, minerals, vitamins, amino acids, fatty acids, secondary metabolites, and antinutrients.

Analysis of variance (ANOVA) was used to test for entry effects both across locations and within each location. Statistical comparisons were performed between the test and control. In addition, mean levels of components were compared to the ranges (minimum to maximum) of values for the nontransgenic reference hybrids and to the ranges for conventional maize published in the Agriculture & Food Systems Institute (AFSI) Crop Composition Database.

Statistical comparisons were made across locations between the test maize and the control maize. The composition data judged suitable for statistical analysis were subjected to across-location ANOVA using the following mixed model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$$

where Y_{ijk} is the observed response for entry i at location j in block k. The overall mean is represented by U, T_i is the entry effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within location, LT_{ij} is the location-by-entry interaction effect, and e_{ijk} is the residual error. Entry was

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regarded as a fixed effect while location, block within location, and location-by- entry interactions were regarded as random effects. The reference hybrids were not included in the across-location analyses to avoid the possibility of the residual error being inflated by any interaction between location and reference hybrids and thus potentially obscuring possible significant differences among the entries of interest.

The composition data judged suitable for statistical analysis were subjected to within-location ANOVA using the following mixed model:

$$Y_{ij} = U + T_i + B_j + e_{ij}$$

where Y_{ij} is the observed response for entry i in block j. The overall mean is represented by U, T_i is the entry effect, B_j is the effect of block, and e_{ij} is the residual error. Entry was regarded a fixed effect, while block was regarded as a random effect. Each within-location analysis included the test, the control, and the three reference hybrids grown at each location.

For each component with data suitable for ANOVA, the SEM was calculated, and, within the context of the model, t-tests were used to assess statistical significance of any differences between the test and the control. Significance was based on the customary alpha level of 0.05, and denominator degrees of freedom were determined by the Kenward-Roger method (Kenward and Roger, 1997.

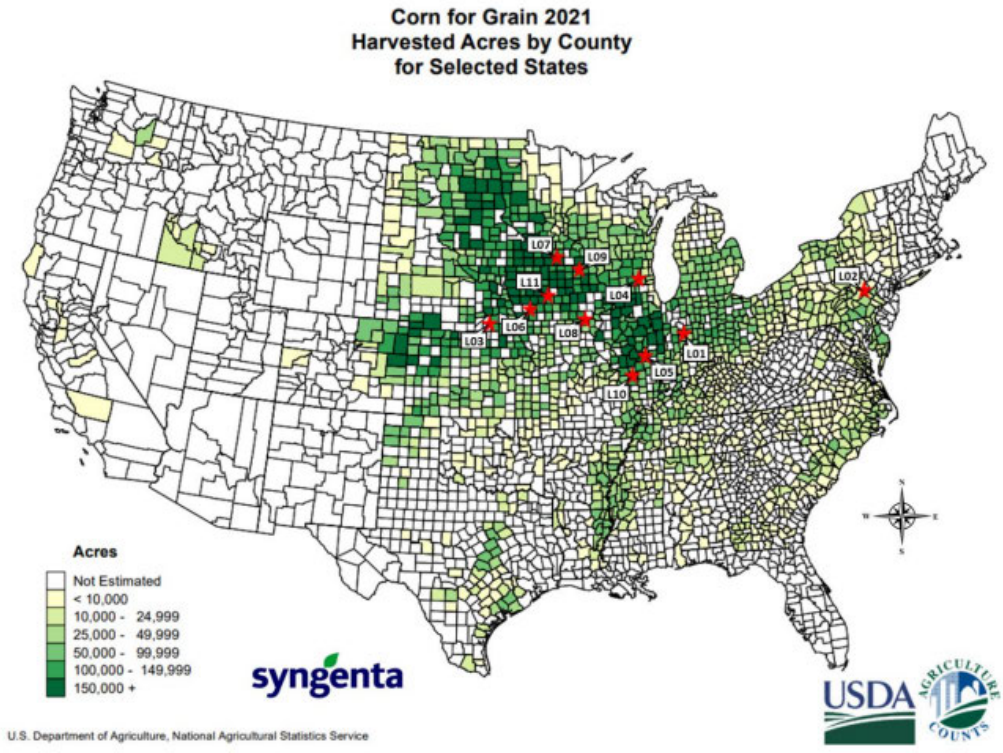

FIGURE 30. Map Trial Locations

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TABLE 21. List of Components Analysed in Maize Forage and Grain

Tissue type	Components				
Forage					
Proximates, fiber, and minerals	moisture protein fat ash total carbohydrates	acid detergent fiber neutral detergent fiber calcium phosphorus			
Grain					
Proximates, fiber, starch, and minerals	moisture[a] protein fat ash total carbohydrates acid detergent fiber neutral detergent fiber total dietary fiber starch calcium	copper iron magnesium manganese phosphorus potassium sodium selenium zinc	Amino acids	alanine arginine aspartic acid cystine glutamic acid glycine histidine isoleucine leucine	lysine methionine phenylalanine proline serine threonine tryptophan tyrosine valine
Fatty acids	8:0 caprylic 10:0 capric 12:0 lauric 14:0 myristic 14:1 myristoleic 15:0 pentadecanoic 15:1 pentadecenoic 16:0 palmitic 16:1 palmitoleic 17:0 heptadecanoic 17:1 heptadecenoic	18:0 stearic 18:1 oleic 18:2 linoleic 18:3 gamma linolenic 18:3 linolenic 20:0 arachidic 20:1 eicosenoic 20:2 eicosadienoic 20:3 eicosatrienoic 20:4 arachidonic 22:0 behenic	Vitamins	β-carotene thiamin riboflavin niacin pyridoxine folic acid α-tocopherol	
Secondary metabolites	ferulic acid furfural inositol *p*-coumaric acid		Anti-nutrients	phytic acid raffinose trypsin inhibitor	

[a]Grain was dried either in the field or mechanically after harvest; therefore, moisture levels were not compared statistically.

(i) Summary of results

Statistically significant differences were detected for four out of nine components in forage: moisture, protein, fat, and total carbohydrates. However, the mean levels of all forage components in the test, including those that were significantly different from the control, were within the ranges for the reference hybrids and the ranges published in the AFSI database.

Out of the 58 quantifiable components in the grain that were compared statistically, the mean levels of 47 components in the test were not significantly different from those in the control. Statistically significant differences in component levels between the test and control were observed for two minerals (copper and manganese), four vitamins (α-tocopherol, β-carotene, pyridoxine, and thiamin), three fatty acids (16:1 palmitoleic, 17:0 heptadecanoic, and 20:1 eicosenoic acids), one secondary metabolite (p-coumaric acid), and one anti-nutrient (raffinose). The mean proportion of 20:1 eicosenoic acid in the test maize was both statistically significantly lower than in the control maize and fell below the lower limit of the reference hybrids range. However, the mean levels of all quantifiable grain components in the test, including that of 20:1 eicosenoic acid, were within the ranges published in the AFSI database.

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(ii) Detailed results

a) Forage - Proximates and fiber

No differences were detected between the test and control in levels of ash, acid detergent fiber (ADF) or neutral detergent fiber (NDF) (Table 22). Differences were detected in levels of moisture, protein, fat, and total carbohydrates. Levels of moisture, protein, and fat were lower in the test relative to the control, while the level of total carbohydrates was higher in the test relative to the control. The mean levels of all proximates and fiber in the test were within the ranges for the reference hybrids and within the ranges reported in the AFSI database.

b) Forage - Calcium and phosphorus

No differences were detected between the test and control in levels of calcium or phosphorus (Table 22). The mean levels of calcium and phosphorus in the test were within the ranges of the reference hybrids and those reported in the AFSI database.

c) Grain - Proximates, fiber, and starch

No differences were detected between the test and control in levels of all proximates, types of fiber, or starch (Table 23). The mean levels of all proximates, types of fiber, and starch in the test were within the ranges of the reference hybrids and the ranges reported in the AFSI database. Grain moisture levels were adjusted by drying, either mechanically or in the field, and were therefore not compared statistically.

d) Grain - Minerals

No differences were detected between the test and control in levels of all quantifiable minerals, except for copper and manganese (Table 24). The mean level of copper was lower in the test relative to the control, while the mean level of manganese was higher in the test relative to the control. Across-location means and statistical comparisons could not be determined for sodium as all values were <LOQ. The mean levels of all quantifiable minerals in the test, including those that were significantly different from the control, were within the ranges of the reference hybrids and the ranges reported in the AFSI database.

e) Grain - Vitamins

No differences were detected between the test and control in levels of folic acid, niacin, or riboflavin (Table 25). Differences were detected in levels of α-tocopherol, β-carotene, pyridoxine, and thiamine. Levels of α-tocopherol and pyridoxine were lower in the test relative to the control, while levels of β-carotene and thiamin were higher in the test relative to the control. The mean levels of all quantifiable vitamins in the test, including those that were significantly different from the control, were within the ranges of the reference hybrids and the ranges reported in the AFSI database.

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f) Grain - Amino acids

No differences were detected between the test and control in levels of any amino acids (Tables 26 and 27). The mean levels of all amino acids in the test were within the ranges of the reference hybrids and those reported in the AFSI database.

g) Grain - Fatty acids

No differences were detected between the test and control in the proportions of 16:0 palmitic, 18:0 stearic, 18:1 oleic, 18:2 linoleic, 18:3 linolenic, 20:0 arachidic, or 22:0 behenic acids (Table 28). Differences were detected in the proportions of 16:1 palmitoleic, 17:0 heptadecanoic, and 20:1 eicosenoic acids. Proportions of 16:1 palmitoleic and 17:0 heptadecanoic acids were higher in the test relative to the control, while the proportion of 20:1 eicosenoic acid was lower in the test relative to the control.

Across-location means and statistical comparisons could not be determined for proportions of 8:0 caprylic, 10:0 capric, 12:0 lauric, 14:0 myristic, 14:1 myristoleic, 15:0 pentadecanoic, 15:1 pentadecenoic, 17:1 heptadecenoic, 18:3 gamma linolenic, 20:2 eicosadienoic, 20:3 eicosatrienoic, and 20:4 arachidonic acids, as most or all values were <LOQ.

Although the mean proportion of 20:1 eicosenoic acid fell below the lower limit of the reference hybrids range, it was still within the range of values reported in the AFSI database. The mean proportions of the other quantifiable grain components in the test, including those of 16:1 palmitoleic and 17:0 heptadecanoic acids, were within the ranges for the reference hybrids and the ranges reported in the AFSI database.

h) Grain - Secondary metabolites and anti-nutrients

No differences were detected between the test and control in levels of all quantifiable secondary metabolites and anti-nutrients, except for *p*-coumaric acid and raffinose (Table 29). The levels of *p*-coumaric acid and raffinose were lower in the test relative to the control. The mean levels of all quantifiable secondary metabolites and anti-nutrients in the test, including those that were significantly different from the control, were within the ranges of the reference hybrids and the ranges reported in the AFSI database.

In Conclusion:

- Forage and grain from Event MZIR260 maize are not materially different in nutrient composition from forage and grain of the nontransgenic, near isogenic comparator or conventional maize.

- Most of the forage and grain components in the test did not differ from the control and where differences did occur, the mean levels in the test were within ranges considered to be normal for conventional maize.

- No biologically relevant impact was observed on the nutritional status of forage and grain in MZIR260 as a result of the transformation process or the newly introduced proteins.

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TABLE 22. Forage Components: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
Moisture (% of fresh weight)	Control	69.9	60.7–77.8	1.86	***0.0183***
	Test	68.4	56.1–76.3		
	Reference hybrids	70.1	52.7–79.3		
	AFSI (N = 8110)	70.3	46.5–90.7		
Protein (%)	Control	8.99	6.10–11.6	0.276	***0.0212***
	Test	8.39	5.97–10.1		
	Reference hybrids	9.03	2.85–15.7		
	AFSI (N = 7691)	7.57	2.37–16.32		
Fat (%)	Control	2.48	1.17–3.43	0.109	***0.0162***
	Test	2.20	1.43–3.21		
	Reference hybrids	2.71	1.08–5.48		
	AFSI (N = 7691)	2.35	<LOQ–7.5		
Ash (%)	Control	4.20	2.07–6.07	0.391	0.0735
	Test	3.78	1.57–7.72		
	Reference hybrids	4.16	1.56–7.42		
	AFSI (N = 8110)	4.22	0.66–13.2		
Total carbohydrates (%)	Control	84.4	80.9–88.4	0.54	***0.0022***
	Test	85.6	79.6–89.8		
	Reference hybrids	84.1	74.3–90.8		
	AFSI (N = 7691)	85.9	73.3–92.9		
Acid detergent fiber (%)	Control	22.9	12.4–31.5	1.47	0.9463
	Test	22.8	11.7–34.3		

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	Reference hybrids	22.3	10.9–36.7		
	AFSI (N = 7429)	25.7	5.13–47.4		

Component	Entry	Mean	Range	SEM	*p*
Neutral detergent fiber (%)	Control	39.3	22.6–51.4	2.18	0.7880
	Test	38.9	22.1–50.8		
	Reference hybrids	37.7	18.2–58.0		
	AFSI (N = 7429)	41.7	18.3–67.8		
Calcium (ppm)	Control	2310	1100–4210	151.1	0.0950
	Test	1965	886–3310		
	Reference hybrids	2338	532–4970		
	AFSI (N = 6964)	1919	368–5768		
Phosphorus (ppm)	Control	1813	1070–2450	84.6	0.4531
	Test	1764	1240–2490		
	Reference hybrids	1923	1080–4520		
	AFSI (N = 6964)	2007	666–4850		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$.
$p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font.
Component levels are presented on percent dry-weight basis except moisture, which is presented on percent fresh-weight basis.
AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.

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TABLE 23 Grain Proximates, Fiber, and Starch Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
Moisture (% of fresh weight)[a]	Control	13.5	9.61–19.3	–	–
	Test	13.2	9.43–19.3		
	Reference hybrids	12.4	9.15–17.4		
	AFSI (N = 10467)	15.0	5.1–40.7		
Protein (%)	Control	10.1	8.63–11.7	0.31	0.1760
	Test	9.92	7.77–12.1		
	Reference hybrids	9.94	7.34–11.8		
	AFSI (N = 9494)	10.1	5.72–17.3		
Fat (%)	Control	2.72	2.00–3.20	0.055	0.1969
	Test	2.61	2.00–3.17		
	Reference hybrids	3.12	2.02–4.03		
	AFSI (N = 9641)	3.82	1.36–7.83		
Ash (%)	Control	1.25	1.03–1.49	0.025	0.7094
	Test	1.24	0.983–1.48		
	Reference hybrids	1.25	0.957–1.46		
	AFSI (N = 10041)	1.39	0.616–6.28		
Total carbohydrates (%)	Control	85.9	84.1–87.5	0.34	0.1263
	Test	86.2	83.9–88.6		
	Reference hybrids	85.7	83.6–88.9		
	AFSI (N = 9616)	84.7	77.4–89.7		
Acid detergent fiber (%)	Control	4.47	3.25–6.14	0.133	0.6394

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Component	Entry	Mean	Range	SEM	*p*
	Test	4.56	3.19–6.03		
	Reference hybrids	3.77	2.43–6.19		
	AFSI (N = 9313)	3.77	1.41–11.34		
Neutral detergent fiber (%)	Control	12.7	10.3–20.9	0.40	0.4001
	Test	13.2	9.42–18.3		
	Reference hybrids	10.4	7.70–15.3		
	AFSI (N = 9312)	10.4	4.28–24.3		
Total dietary fiber (%)	Control	16.1	11.4–28.3	0.7122	0.9269
	Test	16.2	11.7–26.0		
	Reference hybrids	13.0	9.74–20.1		
	AFSI (N = 6981)	13.1	4.44–35.3		
Starch (%)	Control	69.0	63–76.4	0.60	0.3007
	Test	68.1	58.2–73.7		
	Reference hybrids	70.5	61.3–76.7		
	AFSI (N = 2517)	66.9	26.5–83.7		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$.
$p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font.
Proximate and starch levels presented on percent dry-weight basis except moisture, which is presented on percent fresh-weight basis.
AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.
[a]Grain was dried either in the field or mechanically dried after harvest, so moisture levels were not subjected to ANOVA.

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TABLE 24 Grain Mineral Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
Calcium (ppm)[a]	Control	37.1	30.5–48.8	1.23	0.6520
	Test	36.8	30.6–48.8		
	Reference hybrids	33.6	22.6–43.8		
	AFSI (N = 9309)	41.3	<LOQ–1010		
Copper (ppm)	Control	2.05	1.59–2.64	0.098	***0.0389***
	Test	1.88	1.23–2.46		
	Reference hybrids	1.51	0.996–2.06		
	AFSI (N = 9165)	1.68	<LOQ–21.2		
Iron (ppm)	Control	19.9	15.4–22.2	0.56	0.5311
	Test	20.1	15.6–23.3		
	Reference hybrids	19.4	15.2–23.9		
	AFSI (N = 9190)	19.8	7.31–191		
Magnesium (ppm)	Control	1155	1000–1300	20.8	0.1878
	Test	1130	938–1270		
	Reference hybrids	1143	909–1400		
	AFSI (N = 9194)	1193	594–1940		
Manganese (ppm)	Control	7.05	5.38–8.99	0.325	***0.0001***
	Test	7.73	5.66–10.3		
	Reference hybrids	5.30	3.40–8.70		
	AFSI (N = 9193)	6.32	1.69–14.3		
Phosphorus (ppm)	Control	2926	2430–3460	55.0	0.6435

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Component	Entry	Mean	Range	SEM	*p*
	Test	2900	2190–3390		
	Reference hybrids	2967	2340–3550		
	AFSI (N = 9309)	3126	1300–5520		
Potassium (ppm)	Control	3441	2930–3740	38.6	0.5680
	Test	3467	3060–3880		
	Reference hybrids	3504	3000–4270		
	AFSI (N = 9194)	3655	1810–6030		
Selenium (ppm)[b]	Control	0.176	0.0290–0.327	0.0415	0.7701
	Test	0.186	0.0306–0.554		
	Reference hybrids	0.175	0.0279–0.458		
	AFSI (N = 3481)	0.26	0.03–1.51		
Zinc (ppm)	Control	22.1	17.4–27.4	0.77	0.1068
	Test	22.6	18.2–29.7		
	Reference hybrids	21.5	16.7–27.8		
	AFSI (N = 9194)	22.1	6.5–42.6		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$. $p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font. Mineral levels presented on a dry-weight basis. AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.
[a]Five calcium values, corresponding to one reference hybrid entry from locations L02, L04, and L08, were substituted with LOQ values. The substituted LOQ values for calcium were 22.7–24.2 ppm.
[b]Due to an abundance of selenium values <LOQ occurring among all entries at locations L02, L04, L05, and L08, these four locations were excluded from the across-location statistical analysis (Test: $N = 16$. Control: $N = 16$. Reference hybrids: $N = 48$). Eight selenium values were substituted with LOQ values: three values from two reference hybrid entries from location L01, one value each from the control entry and two reference hybrid entries from location L06, and one value each from the test entry and a reference hybrid entry from location L07. The substituted LOQ values for selenium were 0.0279–0.0306 ppm.
All sodium values were <LOQ, making substitution with the LOQ not appropriate due to the number of substitutions required; therefore, valid calculation of the mean and ANOVA could not be performed on the component.

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TABLE 25 Grain Vitamin Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
α-Tocopherol (mg/100 g)[a]	Control	1.38	0.846–2.52	0.076	***0.0263***
	Test	1.18	0.754–1.63		
	Reference hybrids	1.20	0.562–2.26		
	AFSI (N = 8039)	1.07	<LOQ–6.87		
β-Carotene (mg/100 g)[b]	Control	0.0749	0.0562–0.0883	0.00300	***0.0396***
	Test	0.0820	0.0616–0.108		
	Reference hybrids	0.151	0.0228–0.320		
	AFSI (N = 5412)	0.121	<LOQ–0.581		
Folic acid (mg/100 g)	Control	0.0432	0.0288–0.0610	0.00210	0.1822
	Test	0.0453	0.0317–0.0623		
	Reference hybrids	0.0368	0.0231–0.0651		
	AFSI (N = 8833)	0.066	<LOQ–1.14		
Niacin (mg/100 g)	Control	1.79	1.47–2.30	0.070	0.9357
	Test	1.80	1.43–2.40		
	Reference hybrids	2.28	1.39–3.56		
	AFSI (N = 8371)	2.00	<LOQ–6.6		
Pyridoxine (mg/100 g)	Control	0.593	0.488–0.719	0.0169	***0.0033***
	Test	0.524	0.385–0.626		
	Reference hybrids	0.668	0.433–0.979		
	AFSI (N = 8371)	0.573	<LOQ–1.21		

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Component	Entry	Mean	Range	SEM	*p*
Riboflavin (mg/100 g)	Control	0.157	0.103–0.210	0.0040	0.2246
	Test	0.151	0.109–0.180		
	Reference hybrids	0.154	0.112–0.210		
	AFSI (N = 8734)	0.196	<LOQ–0.735		
Thiamin (mg/100 g)	Control	0.351	0.282–0.460	0.0130	***<0.0001***
	Test	0.375	0.291–0.433		
	Reference hybrids	0.355	0.183–0.515		
	AFSI (N = 8415)	0.357	<LOQ–4.00		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$.
$p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font.
Vitamin levels presented on a dry-weight basis.
AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.
[a]Two α-tocopherol values, each from reference hybrid entries at locations L01 and L07, were substituted with LOQ values. The substituted LOQ values for α-tocopherol were 0.562 mg/100g and 0.605 mg/100g.
[a]One β-Carotene value from a reference hybrid entry at location L06 was substituted with an LOQ value (0.0228 mg/100g).

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TABLE 26 Grain Amino Acid Composition [Alanine–Leucine]: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
Alanine (mg/g)	Control	7.95	6.51–9.89	0.271	0.2216
	Test	7.78	5.25–9.77		
	Reference hybrids	7.63	5.06–9.65		
	AFSI (N = 9289)	7.67	4.00–14.8		
Arginine (mg/g)	Control	4.99	4.03–5.84	0.115	0.7175
	Test	4.95	4.05–6.60		
	Reference hybrids	5.00	3.77–6.61		
	AFSI (N = 9289)	4.62	1.19–7.08		
Aspartic acid (mg/g)	Control	6.55	5.90–8.06	0.166	0.4811
	Test	6.47	4.91–8.02		
	Reference hybrids	6.68	5.04–10.1		
	AFSI (N = 9289)	6.62	2.98–12.08		
Cystine (mg/g)	Control	2.24	1.58–2.77	0.102	0.1727
	Test	2.31	1.64–2.97		
	Reference hybrids	2.18	1.34–2.79		
	AFSI (N = 9288)	2.10	0.95–5.14		
Glutamic acid (mg/g)	Control	19.2	15.7–23.8	0.71	0.2161
	Test	18.8	12.8–24.2		
	Reference hybrids	18.6	12.1–24.1		
	AFSI (N = 9289)	19.2	8.32–35.4		
Glycine (mg/g)	Control	3.85	3.30–4.46	0.088	0.7751
	Test	3.87	3.28–4.84		
	Reference hybrids	3.75	2.73–4.93		
	AFSI (N = 9289)	3.82	1.84–6.85		

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Component	Entry	Mean	Range	SEM	*p*
Histidine (mg/g)	Control	2.77	2.47–3.15	0.064	0.5001
	Test	2.74	2.12–3.20		
	Reference hybrids	2.72	2.07–3.26		
	AFSI (N = 9289)	2.81	1.37–4.56		
Isoleucine (mg/g)	Control	3.57	3.04–4.51	0.116	0.1037
	Test	3.48	2.49–4.35		
	Reference hybrids	3.54	2.41–4.49		
	AFSI (N = 9289)	3.58	1.79–6.92		
Leucine (mg/g)	Control	12.9	10.2–16.6	0.55	0.0842
	Test	12.5	8.16–16.6		
	Reference hybrids	12.5	7.75–17.0		
	AFSI (N = 9289)	12.7	6.04–24.9		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$.
$p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font.
Amino acid levels presented on a dry-weight basis.
AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.

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TABLE 27 Grain Amino Acid Composition [Lysine–Valine]: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
Lysine (mg/g)	Control	2.85	2.33–3.37	0.061	0.9338
	Test	2.85	2.25–3.87		
	Reference hybrids	2.89	2.20–4.09		
	AFSI (N = 9280)	2.89	1.27–6.68		
Methionine (mg/g)	Control	1.97	1.51–2.37	0.090	0.4335
	Test	1.95	1.45–2.62		
	Reference hybrids	1.92	1.17–2.42		
	AFSI (N = 9286)	2.05	0.89–4.68		
Phenylalanine (mg/g)	Control	5.15	4.27–6.50	0.180	0.1102
	Test	5.01	3.46–6.36		
	Reference hybrids	5.09	3.33–6.74		
	AFSI (N = 9289)	5.17	2.44–9.30		
Proline (mg/g)	Control	8.89	7.43–10.9	0.307	0.6219
	Test	8.83	6.31–11.1		
	Reference hybrids	8.94	6.61–10.7		
	AFSI (N = 9289)	9.02	4.62–17.5		
Serine (mg/g)	Control	4.92	4.29–5.82	0.146	0.4317
	Test	4.87	3.57–5.95		
	Reference hybrids	4.84	3.45–6.02		
	AFSI (N = 9289)	4.86	1.52–7.69		
Threonine (mg/g)	Control	3.63	3.24–4.18	0.088	0.6914
	Test	3.61	2.85–4.28		
	Reference hybrids	3.53	2.75–4.33		
	AFSI (N = 9289)	3.61	1.73–6.66		
Tryptophan (mg/g)	Control	0.793	0.663–0.903	0.0196	0.3934
	Test	0.785	0.667–0.910		
	Reference hybrids	0.772	0.576–0.901		

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Component	Entry	Mean	Range	SEM	*p*
	AFSI (N = 9287)	0.72	0.27–2.15		
Tyrosine (mg/g)	Control	4.47	3.59–5.52	0.162	0.6407
	Test	4.43	3.29–5.76		
	Reference hybrids	4.25	3.06–5.37		
	AFSI (N = 9289)	3.46	1.03–7.34		
Valine (mg/g)	Control	4.73	4.24–5.62	0.118	0.1440
	Test	4.63	3.52–5.55		
	Reference hybrids	4.67	3.49–5.80		
	AFSI (N = 9289)	4.71	2.66–8.55		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$.
$p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font.
Amino acid levels presented on a dry-weight basis.
AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.

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TABLE 28. Grain Fatty Acid Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
16:0 Palmitic (% of total)	Control	16.1	15.5–17.2	0.12	0.2731
	Test	16.3	15.3–17.8		
	Reference hybrids	15.5	13.0–18.0		
	AFSI (N = 6950)	12.6	6.81–26.6		
16:1 Palmitoleic (% of total)	Control	0.143	0.128–0.160	0.0025	***<0.0001***
	Test	0.157	0.141–0.168		
	Reference hybrids	0.133	0.103–0.184		
	AFSI (N = 6586)	0.137	<LOQ–0.453		
17:0 Heptadecanoic (% of total)	Control	0.0847	0.0768–0.0901	0.00130	***0.0191***
	Test	0.0872	0.0767–0.0954		
	Reference hybrids	0.0799	0.0684–0.0994		
	AFSI (N = 6586)	0.0860	<LOQ–0.203		
18:0 Stearic (% of total)	Control	2.26	1.86–2.70	0.092	0.7474
	Test	2.25	1.85–2.76		
	Reference hybrids	1.97	1.47–2.32		
	AFSI (N = 6950)	1.89	1.02–3.83		
18:1 Oleic (% of total)	Control	21.4	19.5–24.2	0.40	0.3540
	Test	21.5	19.2–23.5		
	Reference hybrids	25.4	19.9–32.5		
	AFSI (N = 6950)	26.3	16.4–42.8		
18:2 Linoleic (% of total)	Control	57.1	54.2–59.6	0.54	0.0607
	Test	56.7	53.4–59.6		
	Reference hybrids	54.4	46.9–60.7		
	AFSI (N = 6950)	56.8	34.3–67.7		
18:3 Linolenic (% of total)	Control	2.09	1.88–2.45	0.041	0.0568
	Test	2.16	1.95–2.46		
	Reference hybrids	1.78	1.29–2.23		

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Component	Entry	Mean	Range	SEM	*p*
	AFSI (N = 6950)	1.37	0.55–2.33		
20:0 Arachidic (% of total)	Control	0.392	0.350–0.458	0.0117	0.0725
	Test	0.395	0.350–0.481		
	Reference hybrids	0.385	0.309–0.468		
	AFSI (N = 6612)	0.415	0.267–0.993		
20:1 Eicosenoic (% of total)	Control	0.185	0.178–0.197	0.0011	***0.0367***
	Test	0.182	0.175–0.190		
	Reference hybrids	0.218	0.187–0.269		
	AFSI (N = 6612)	0.262	<LOQ–1.95		
22:0 Behenic (% of total)	Control	0.188	0.157–0.225	0.0052	0.6653
	Test	0.190	0.156–0.232		
	Reference hybrids	0.180	0.120–0.272		
	AFSI (N = 6612)	0.175	<LOQ–0.417		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$.
$p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font.
AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.
Because the majority or all values were <LOQ for 12 other fatty acids, substitution with the LOQ was not appropriate due to the number or distribution of substitutions required; therefore, valid calculation of the mean and ANOVA could not be performed.

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TABLE 29. Grain Secondary Metabolite and Anti-Nutrient Composition: Across-Location Comparison of Event MZIR260 Maize (Test) and Nontransgenic, Near-Isogenic Maize (Control)

Component	Entry	Mean	Range	SEM	*p*
Ferulic acid (ppm)	Control	2878	2400–3650	53.0	0.7747
	Test	2860	2510–3330		
	Reference hybrids	2212	1650–3330		
	AFSI (N = 8749)	2280	292–4397		
Inositol (ppm)	Control	2196	1650–2680	64.8	0.7176
	Test	2228	1450–2950		
	Reference hybrids	2117	1410–2730		
	AFSI (N = 3192)	2517	1116–4750		
p-Coumaric acid (ppm)	Control	317	259–377	8.9	***0.0239***
	Test	299	250–376		
	Reference hybrids	231	129–346		
	AFSI (N = 8748)	226	<LOQ–820		
Phytic acid (%)	Control	0.780	0.583–1.00	0.0258	0.9542
	Test	0.782	0.504–1.05		
	Reference hybrids	0.782	0.499–1.08		
	AFSI (N = 9140)	0.87	<LOQ–1.94		
Raffinose (%)[a]	Control	0.112	0.0571–0.152	0.0102	***0.0115***
	Test	0.100	0.0564–0.142		
	Reference hybrids	0.176	0.0782–0.273		
	AFSI (N = 8733)	0.177	<LOQ–0.466		
Trypsin inhibitor (TIU/mg)	Control	1.31	0.799–1.75	0.061	0.4761
	Test	1.26	0.88–1.60		
	Reference hybrids	1.17	0.677–1.68		
	AFSI (N = 5696)	3.36	<LOQ–8.42		

Test: $N = 32$. Control: $N = 32$. Reference hybrids: $N = 96$.
$p = \Pr > |t|$. Results considered significantly different ($p < 0.05$) are displayed in bold, italic font.
Units are shown on a dry-weight basis.
AFSI: N is the number of AFSI (2023) values used to calculate the mean and excludes values <LOQ.
[a]Due to the distribution of substitutions required for raffinose values <LOQ, three for the test entry and one for the control entry, location L07 was excluded from the across-location statistical analysis (Test: $N = 28$. Control: $N = 28$. Reference hybrids: $N = 84$). Two values for the test entry at location L02 were substituted with LOQ values. The substituted LOQ values for raffinose were 0.0564% and 0.0566%.
All values for furfural were <LOQ, and substitution with the LOQ was not appropriate due to the number of substitutions required; therefore, valid calculation of the mean and ANOVA could not be performed.

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Section 3.06 Annex III References

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Article IV. APPENDICES (Company Data to Support this Application)

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