Supporting document 1

Safety assessment – Application A1262

Food derived from insect-protected corn line MON95275

Executive summary

Background

Application A1262 seeks approval for the sale and use of food derived from genetically modified (GM) corn line MON95275 that is protected against coleopteran insect pests, primarily western corn rootworm (WCR).

Protection against coleopteran insect pests is conferred by the expression in the plant of three novel substances: the Mpp75Aa1.1 protein (encoded by the *mpp75Aa1.1* gene) from *Brevibacillus laterosporus*, the Vpb4Da2 protein (encoded by the *vpb4Da2* gene) from *Bacillus thuringiensis* and DvSnf7, and a double stranded RNA (dsRNA) that specifically silences the WCR *DvSnf7* gene via RNA interference (RNAi). These novel substances cause midgut damage specifically in corn rootworm larvae. While FSANZ has assessed and approved numerous insecticidal proteins with a similar mechanism of action in a variety of plant species, the *mpp75Aa1.1* and *vpb4Da2* genes have not previously been assessed by FSANZ. DvSnf7 dsRNA has been previously assessed by FSANZ (Application A1097).

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

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

History of use

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

Molecular characterisation

The genes encoding Mpp75Aa1.1 (*mpp75Aa1.1*) and Vpb4Da2 (*vpbDda2*), along with the DNA sequences encoding DvSnf7 dsRNA (*DvSnf7.1*) were introduced into corn line MON95275 via *Agrobacterium*-mediated transformation. This transformation also introduced a selectable marker cassette that was subsequently removed using the Cre/*lox* recombination system.

Detailed molecular analyses of corn line MON95275 indicate that a single copy of each of the linked *mpp75Aa1.1*, *vpb4Da2*, and *DvSnf7.1* cassettes is present at a single insertion site in the genome. There are no extraneous plasmid sequences, selectable marker cassettes, Cre recombinase, nor antibiotic resistance marker genes, present in this line.

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

Characterisation and safety assessment of new substances

Newly expressed proteins

Mpp75Aa1.1 and Vpb4Da2 are newly expressed proteins present in MON95275. They are expressed at a low level in grain, and at a high level in leaf tissue. Bioinformatic studies confirmed a lack of any significant amino acid sequence similarity to known protein toxins or allergens. Laboratory studies demonstrated the Mpp75Aa1.1 and Vpb4Da2 proteins are susceptible to the action of digestive enzymes and would be thoroughly degraded before being absorbed during passage through the gastrointestinal tract. The proteins are also susceptible to heat denaturation and degradation at the high temperatures typically used in food processing. Taken together, the evidence supports the conclusion that Mpp75Aa1.1 and Vpb4Da2 are not toxic or allergenic in humans.

DvSnf7 dsRNA

The 240 bp DvSnf7 dsRNA expressed in MON95275 triggers RNAi specifically in corn rootworms from the genus *Diabrotica*. The safety of the DvSnf7 dsDNA has been previously assessed by FSANZ (Application A1097). The available data do not indicate the dsRNA expressed in this line possess different characteristics, or is likely to pose a greater risk, than other RNAi mediators naturally present in corn.

Compositional analyses

Detailed compositional analyses were performed on MON95275. Statistically significant differences were found between grain from MON95275 and the control for 7 of the 68 analytes evaluated, however these differences were small and all within the range established for existing commercial non-GM corn cultivars. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in grain from MON95275 compared to non-GM corn cultivars available on the market.

Conclusion

No potential public health and safety concerns have been identified in the assessment of insect-protected corn line MON95275. On the basis of the data provided in the present application and other available information, food derived from MON95275 is considered to be as safe for human consumption as food derived from non-GM corn cultivars.

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List of Abbreviations

Abbreviation	Description
ADF	acid detergent fibre
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
CI	confidence interval
COMPARE	COMprehensive Protein Allergen REsource
Cry	crystal protein
DNA	deoxyribonucleic acid
dw	dry weight
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvyl-3-shikimatephosphate synthase
FASTA	fast alignment search tool – all
FSANZ	Food Standards Australia New Zealand
g	gram
GM	genetically modified
h	hours
HFCS	high fructose corn syrup
kDa	kilodalton
LB	left border of T-DNA
LOD	limit of detection
LOQ	limit of quantitation
mg	milligram
min	minutes
mRNA	messenger RNA

Abbreviation	Description
MT	million tons
NCBI	National Centre for Biotechnology Information
NDF	neutral detergent fibre
ng	nanogram
NGS	next generation sequencing
ns	not significant
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
PCR	polymerase chain reaction
RB	right border of T-DNA
RNA	ribonucleic acid
RNAi	RNA interference
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
TDF	total dietary fibre
T-DNA	transfer DNA
µg	microgram
UTR	untranslated region
WCR	western corn rootworm

1   Introduction

FSANZ received an application from Bayer CropScience Proprietary Limited to vary Schedule 26 in the *Australia New Zealand Food Standards Code*. The variation is to include food from a new genetically modified (GM) corn line MON95275, with the OECD Unique Identifier MON-95275-7. This corn line is protected against coleopteran insect pests, primarily western corn rootworm (WCR; *Diabrotica virgifera virgifera*).

Protection from coleopteran insect pests is achieved by expression of the insecticidal Mpp75Aa1.1 and Vpb4Da2 proteins, encoded by the bacterial genes *mpp75Aa1.1* and *vpb4Da2* from *Brevibacillus laterosporus* and *Bacillus thuringiensis*, respectively. MON95275 also contains DvSnf7.1 double-stranded RNA (dsRNA). The introduced DNA sequences are derived from the *Snf7* gene from *Diabrotica virgifera virgifera*. When consumed by WCR larvae, DvSnf7 dsRNA triggers the insect's RNA interference (RNAi) machinery, leading to insect death.

A1262 is the first application to assess the Mpp75Aa1.1 and Vpb4Da2 proteins. However, the DvSnf7 dsRNA has been previously assessed and approved by FSANZ (Application A1097; FSANZ 2015)

If approved, food derived from MON95275 corn line may enter the Australian and New Zealand food supply as imported food products.

2   History of use

2.1 Host organism

The host organism is corn (*Zea mays*) which is also referred to as maize. The inbred corn line LH244 was used as the parental variety for the genetic modification described in this application. This variety is a dent cultivar, which is a medium season yellow corn line that grows well in the central regions of the United States corn belt. Corn line LH244, or its cross with proprietary elite inbred line HCL617, were used as the conventional control for the purposes of comparative assessment with MON95275.

Corn was one of the first plants to be cultivated by humans (Ranum et al. 2014) and is now the world's dominant cereal crop, with global production of 1,134 MT[1] in 2020/21, ahead of wheat (773 MT) and rice (496 MT) (USDA 2022). Due to its economic importance, corn has been the subject of extensive study.[2]

The United States is the world's largest producer of corn, producing 360 MT in 2020/21 (USDA 2022). Relatively small quantities of corn are grown in Australia and New Zealand - in 2021 these amounted to 0.306 and 0.209 MT respectively (FAOSTAT 2021). Of the corn grown in the United States and Canada, an estimated 92% and ~80%, respectively, is GM.[3,4] No GM corn is currently grown commercially in Australia or New Zealand.

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[1] million tons

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

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

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

To supplement their limited local production of corn, Australia and New Zealand import both corn grain and processed corn products. For example, in 2021 the imported quantities of corn flour into Australia and New Zealand were 11,626 and 1,284 tonnes respectively, while imports of corn oil totalled 1,106 and 122 tonnes respectively (FAOSTAT 2021).

Corn has a long history of safe consumption as food by humans[5]. Food products derived from processing of corn kernels include corn flour, meal, oil, starch and sweeteners such as high fructose corn syrup (HFCS). In Australia and New Zealand, corn starch is used in dessert mixes and canned foods and HFCS is used in breakfast cereals, baking products, corn chips and extruded confectionary.

2.2 Donor organisms

2.2.1 Brevibacillus laterosporus

The source of the *mpp75Aa1.1* gene is the bacterial species *Brevibacillus laterosporus*, previously known as *Bacillus laterosporus* (Shida et al. 1996; Panda et al. 2014). This gram-positive, endospore-forming bacterium is ubiquitous in a number of environments including soil, seawater and freshwater (Ruiu 2013). Although *B. laterosporus* does not have any documented commercial use in food, it has been isolated from a range of foods including milk, cheese, honey and beans (Sarkar et al. 2002; Iurlina and Fritz 2005; Panda et al. 2014), and has been used as a probiotic (Hong et al. 2005). Various *B. laterosporus* strains exhibit pathogenicity to insects and other invertebrates (Ruiu 2013), and *B. laterosporus* strain 1951 has been approved for agricultural pest control in New Zealand (NZ EPA 2022). This species is not pathogenic or allergenic to humans, and its widespread presence in the environment, including in food, provides a history of safe human exposure.

2.2.2 Bacillus thuringiensis

The source of the *vbp4Da2* gene is the bacterium *Bacillus thuringiensis*. *B. thuringiensis* is a gram-positive, endospore-forming bacterium, ubiquitous in soil and considered non-pathogenic to humans (Palma et al. 2014). *B. thuringiensis* expresses a number of insecticidal proteins, including the well-characterised Cry proteins, and various *B. thuringiensis* strains have a long history of use for the control of agricultural insect pests[6] (Nester et al. 2002; CERA 2011). Currently, there are approximately 44 biopesticide products based on *B. thuringiensis* registered in Australia (APVMA 2022) and 10 in New Zealand (ACVM 2022).

Its use in agriculture, along with its ubiquitous presence in soil and on vegetation, means that *B. thuringiensis* has a long history of being safely consumed in food. Despite this, its close relationship with the species *Bacillus cereus*, which contains a number of strains pathogenic to humans, and the presence of enterotoxin genes in some strains (Ehling-Schulz et al. 2019; Biggel et al. 2022), has led to concern that *B. thuringiensis* residues may cause diarrheal illness in humans (Bonis et al. 2021). However, current evidence suggests the mechanism of action that underlies disease in certain *B. cereus* strains is not linked to strains of *B. thuringiensis* used in agriculture (Raymond and Federici 2017; Biggel et al. 2022).

2.2.3 Diabrotica virgifera virgifera

The non-coding *DvSnf7.1* DNA sequence matches a portion of the sequence of the *DvSnf7* gene from *Diabrotica virgifera virgifera*. *D. virgifera* is a beetle (order Coleoptera; family

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

Chrysomelidae) native to North America and responsible for severe yield losses in corn crops throughout the United States (Gray et al. 2009; Coates et al. 2023). *D. virgifera* larvae feed on corn roots, causing both physiological damage to plants as a result of impaired water and nutrient absorption, and harvesting difficulties as a result of plant lodging[7]. There are no reports of any direct effects of the insect on humans.

2.2.4   Other organisms

Genetic elements from several other organisms have been used in the genetic modification of MON95275 (refer to Table 1). These genetic elements are non-coding sequences and are used to regulate the expression of *mpp75Aa1.1*, *vpb4Da2* and *DvSnf7*.

3   Molecular characterisation

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

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

3.1 Transformation method

To create the MON95275 corn line, the inbred LH244 corn line was transformed using the plasmid PV-ZMIR525664 (Figure 1). The *cp4 epsps* selectable marker was subsequently removed using the Cre/*lox* recombination system in an intermediary line. The methodology is outlined in the flowchart in Appendix 1 and summarised below.

Transformation of the LH244 line was achieved by co-culturing immature embryos excised from a post-pollinated corn ear with *Agrobacterium tumefaciens* containing the PV-ZMIR525664 plasmid (Siderov and Duncan 2009). Immature embryos were then placed on selective media containing glyphosate and carbenicillin. Glyphosate inhibits the growth of untransformed plant cells, while carbenicillin suppresses the growth of excess *Agrobacterium*.

After the transformed embryos reached the callus stage, the calli were placed on media to encourage shoot and root development. Rooted plants (R0) with normal phenotypes were transferred to soil and self-pollinated to produce R1 seed. R1 plants were screened using polymerase chain reaction (PCR) and Southern blot analysis to identify R1 plants carrying the transfer DNA (T-DNA) but not the vector backbone (Figure 1). R1 plants that were homozygous for T-DNA and lacked vector backbone were self-pollinated, giving rise to R2 seed.

Selected R2 plants were crossed with a corn line expressing Cre recombinase protein, which was developed separately with the plasmid vector PV-ZMOO513642 ([Appendix 2](#)). R2 plants contain the *cp4 epsps* expression cassette, which is part of the T-DNA inserted during transformation and is flanked by *lox*P sites (Figure 1). The Cre/*lox* recombination system allows the site-specific recombination of two *lox*P sites (Gilbertson 2003; Zhang et al. 2003). The introduction of Cre recombinase in the R2 x Cre recombinase expressing line enabled

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[7] The bending over of the stems near ground level.

the removal of the *cp4 epsps* expression cassette in the F1 generation. Subsequently, the *cre* gene and associated PV-ZMOO513642 sequences were segregated away from the genes of interest by conventional breeding. Progeny were screened for the absence of the *cp4 epsps* expression cassette, the *cre* gene and other sequences derived from PV-ZMOO513642.

Subsequent generations were further screened using standard molecular biology techniques, allowing selection of plants with the *DvSnf7.1*, *mpp75Aa1.1* and *vpb4Da2* expression cassettes, but without unintended DNA insertions. Following the evaluation of insert integrity, trait efficacy, phenotypic characteristics and agronomic performance, corn line MON95275 was selected.
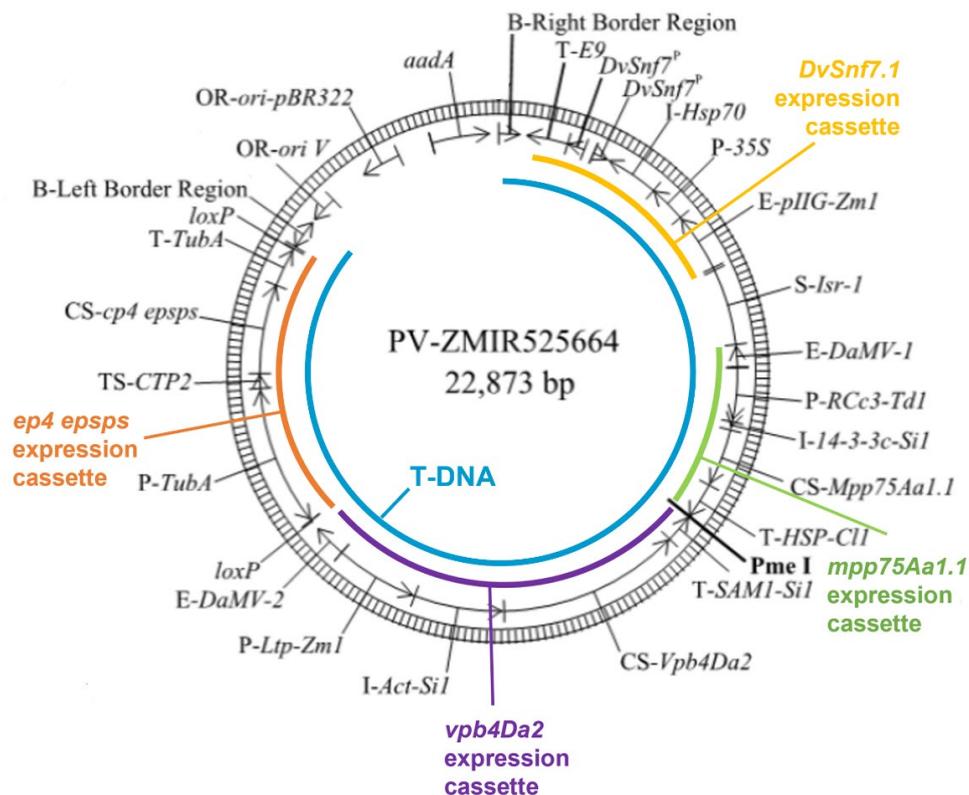
Figure 1. *Map of the PV-ZMIR525664 transformation plasmid. The T-DNA (blue) contains the cp4 epsps (orange), vpb4Da2 (purple), mpp75Aa1.1 (green) and DvSnf7.1 (yellow) expression cassettes. The vector backbone contains two origins of replication (ori pBR322, ori V) and the aadA antibiotic resistance gene. The vector backbone is not incorporated into the plant during transformation but is required for preparing the plasmid, passaging through standard laboratory Escherichia coli (E. coli) and into the Agrobacterium.*

3.2 Detailed description of inserted DNA

Corn line MON95275 contains T-DNA from the PV-ZMIR525664 plasmid (Figure 1) and includes the *mpp75Aa1.1*, *vbp4Da2*, and *DvSnf7.1* expression cassettes. The *cp4 epsps* selectable marker cassette, which confers tolerance to the herbicide glyphosate, was also part of the originally inserted T-DNA, but was removed using Cre/*lox* recombination and is not present in MON95275.

Information on the genetic elements in the T-DNA used for transformation is summarised in Table 1. Additional detail, including intervening sequences used to assist with cloning,

mapping and sequence analysis, can be found in Appendix 2.

Table 1: Expression cassettes contained in the T-DNA of PV-ZMIR525664

	Promoter	Enhancer/ Regulatory sequence	Coding sequence	Terminator	Notes
DvSnf7.1 cassette	35S promoter from Cauliflower mosaic virus (CaMV)	Optimised enhancer from the *Zea mays pllG* gene Intron and flanking exon sequence of the *Zea mays hsp70* gene	Two 240 nt fragments of the *DvSnf7* gene from *D. virgifera*	3'UTR from the *rbcS* gene from *Pisum sativum* (pea)	150 nt intervening sequence present between *DvSnf7* inverted repeats to allow hairpin formation
mpp75Aa1.1 cassette	Promoter of *RCc3* gene from *Tripsacum dactyloides* (Eastern gamma grass)	Enhancer sequence from the promoter region of Dalia Mosaic virus (*DaMV-1*) Intron from a putative *14-3-3c* gene from *Setaria italica*	*mpp75Aa1.1* from *B. laterosporus*	3'UTR of *Hsp* gene from *Coix lacryma-jobi* (adlay millet)	Codon-optimised sequence with N-terminal truncation (23 aa membrane transiting sequence)
vpb4Da2 cassette	Promoter of lipid transfer protein gene (*Ltp*) from *Zea mays* (corn)	Enhancer sequence from the promoter region of Dalia Mosaic virus (*DaMV-2*) Intron from an actin gene (*Act*) from *Setaria italica*	*vpb4Da2* from *B. thuringiensis*	3'UTR of the *SAM1* gene from *Setaria italica*	Full length native protein
cp4 epsps cassette	Promoter, 5'UTR and intron from the α-tubulin gene family (*TubA*) from *Oryza sativa* (rice)	Included with promoter	*cp4 epsps* from *Agrobacterium* sp. strain CP4	3' UTR from the α-tubulin gene family (*TubA*) from *Oryza sativa* (rice)	Confers glyphosate tolerance. Removed from MON95275 by Cre/*lox* recombination (Section 3.1)

3.3 Development of the corn line from the original transformation

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of MON95275
- ensuring that the MON95275 event is incorporated into elite lines for commercialisation.

The breeding history of MON95275, showing the generations used for characterisation studies, is shown in Figure 2. Table 2 indicates the specific generations used in the various analyses of MON95275. As controls, either the non-GM parental line LH244, or the non-GM cross LH244 x HCL617 were used (Table 2), based on the most appropriate genetic background for the generation being analysed.

RO
⊗
R1
⊗
F1 ← × LH244 Cre R2
⊗
F2[4]
⊗
F3
⊗
F4[1,2] × HCL617 → **F4F1**[1]
⊗
F5[1] × HCL617 → **F5F1**[1]
⊗
F6[1,3]

⊗ Designates self-pollination
[1] Generations used to confirm insert stability
[2] Generation used for molecular characterisation
[3] Generation used for commercial development of MON95275
[4] Generation screened for plants lacking the *cre* gene. Only plants lacking *cre* were self-pollinated to create F3 plants lacking *cre*.

Figure 2. *Breeding path used in the characterisation of MON95275. Bolded text indicates generations used to confirm insert stability and molecular characterisation.*

Table 2: MON95275 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Number of integration sites	Section 3.4.1	F4	LH224
Absence of backbone and other sequences	Section 3.4.2	F4	LH224
Insert integrity and site of integration	Section 3.4.3	F4	LH224
Genetic stability	Section 3.4.4.1	F4, F4F1, F5, F5F1, F6	LH244, LH244 x HCL617
Mendelian inheritance	Section 3.4.4.2	F4F2, F4F3, F4F4	N/A
Expression of phenotype over several generations	Section 3.4.4.2	F4, F4F1, F5, F5F1, and F6	LH244 x HCL617
Compositional analysis	Section 5	F5F1	LH244 x HCL617

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

A range of analyses were undertaken to characterise the genetic modification in MON95275. These analyses focused on the nature and stability of the insertion and whether any unintended re-arrangements or products may have occurred as a consequence of the transformation procedure.

Genomic DNA from MON95275 (F4 generation) and from the conventional control (LH244) was sequenced using next generation sequencing (NGS). This method generates ~150 bp short sequence reads which are randomly distributed throughout the genome and in sufficient number to ensure the genomes are covered comprehensively. In addition, the

transformation plasmid PV-ZMIR525664 was sequenced to serve as a reference. To assess the sensitivity of the NGS method, plasmid DNA was spiked and sequenced. Sufficient sequence reads were obtained to cover the entire genomes of MON95275 and the LH244 control, with a depth of coverage ≥75x and an adequate level of sensitivity[8].

3.4.1   Number of integration site(s)

NGS reads from MON95275 (F4) that mapped to the intended T-DNA insert region were analysed and two unique insert-flank junction sites were identified. Each comprised the inserted T-DNA border sequence joined to a flanking sequence in the corn genome. This indicates that a single copy of the intended DNA insert has been integrated into the genome of MON95275 (Figure 3). No junction sequences were detected in the LH244 control.
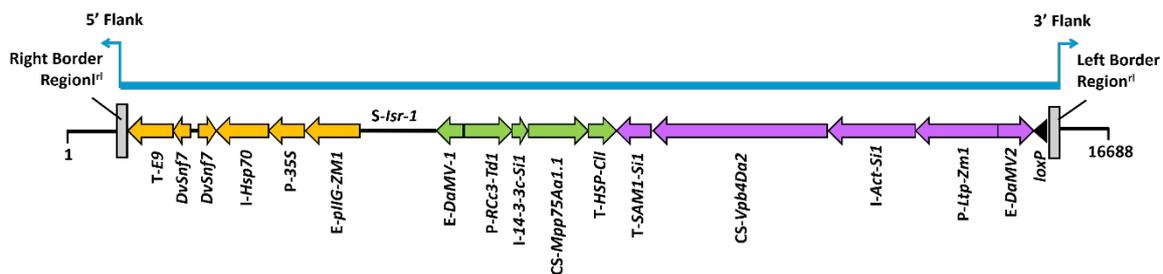
Figure 3. *Insert and flanking sequences present in MON95275. Colouring of cassettes matches that in Figure 1.* **rl** *– both the Left and Right Border regions in MON95275 are truncated compared to PV-ZMIR525664.* **T** *– terminator*; **I** *– intron*; **P** *– promoter*; **E** *– enhancer*; **CS** *– coding sequence*; **S** *- spacer*

3.4.2   Absence of backbone and other sequences

NGS reads from MON95275 (F4) and the PV-ZMIR525664 transformation plasmid were aligned. No reads mapped to any of the sequences from the selectable marker cassette (P-*TubA*, TS-*CTP2*, CS-*cp4 epsps*, and T-*TubA*), with the exception of a single pair of reads which aligned to the P-*TubA* promoter sequence. This single pair of reads is not representative of a true insertion into MON95275 and is instead likely a result of contamination during library preparation. In addition, a single *lox*P site remained in MON95275, as expected. These results confirm the removal of the selectable marker cassette using the Cre/*lox* recombination system.

No reads were detected for the vector backbone sequence *aadA*, though a small number of reads were mapped to the backbone sequences OR-*ori V* and OR-*ori-pBR322* (see Figure 1). This low number of reads does not indicate the presence of backbone sequence in MON95275 and is likely due to the presence of environmental bacteria in the genomic DNA prepared for NGS, as has been previously described (Yang et al. 2013; Zastrow-Hayes et al. 2015). The results of the alignment confirmed there was no integration of backbone sequences into MON95275, including any antibiotic resistance genes.

The LH244 control contained many reads that mapped to the T-DNA elements I-*Hsp70*, E-*pIIG-Zm1* and P-*LtpZm1.* However, these alignments were due to the presence of endogenous sequences in the corn genome that are homologous to these T-DNA encoded elements. As noted above, no junctions between plasmid and genomic DNA were identified

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[8] The NGS method was sufficiently sensitive to detect 100% of the spiked plasmid when present at 1/10th of a copy per genome equivalent or greater. Additionally, reads were mapped to a single copy of an endogenous gene and the depth of coverage for MON95275 and the controls was comprehensive.

in the LH244 control, providing further evidence that these reads arose from endogenous sequences.

During the breeding process used to develop MON95275, the R2 generation was crossed with a corn line expressing a Cre recombinase protein (Figure 2). This corn line was generated using the PV-ZMOO513642 plasmid (Appendix 3). Subsequent generations were screened for the absence of the *cre* gene and other PV-ZMOO513642 sequences. To confirm their absence in MON95275 (F4), reads from MON95275 genomic DNA were aligned with the PV-ZMOO513642 plasmid sequence. As a control, the PV-ZMOO513642 plasmid sequence was also aligned to the LH244 control reads.

The results showed that several reads aligned to the LB region and the P-*35S* promoter sequence of PV-ZMOO513642, which was expected as PV-ZMOO513642 and PV-ZMIR525664 share these sequences, and both regions from PV-ZMIR525664 are present in MON95275 (Figure 3). A small number of reads mapped to the promoter element *Ract1*, which is homologous to a maize sequence in the LH244 background, and as such is not indicative of the presence of PV-ZMOO513642 sequences in MON95275. Additionally, a few reads aligned with the OR-*ori-pBR322* sequence, the OR-*ori V* sequence, and other sequences in the PV-ZMOO513642 backbone used during cloning. As with the detection of backbone sequences from PV-ZMIR525664 noted previously, these alignments are most likely due to the presence of DNA from environmental bacteria in the tissues used for genomic DNA preparation. These results indicate that MON95275 (F4) and subsequent generations do not contain the *cre* gene or other sequences from the PV-ZMOO513642 plasmid.

3.4.3   Insert integrity and site of integration

Locus-specific PCR and DNA sequence analysis of seed-derived genomic DNA from MON95275 showed that a single copy of the T-DNA from PV-ZMIR525664 was integrated into the host genome and the organisation of the genetic elements within the insert is as expected. Alignment of the MON95275 insert sequence with the plasmid T-DNA showed that there was a single nucleotide difference between MON95275 and the plasmid in the non-coding intervening sequence between *S-lsr-1* and *E-DaMV-1* (refer to Appendix 2). Both border regions in MON95725 were also truncated relative to these regions in PV-ZMIR525664. Neither of these changes would have a functional impact on the expression of the inserted cassettes. No deletions, insertions, mutations or rearrangements of the expression cassettes were detected. As expected, the selectable marker cassette (P-*TubA*, TS-*CTP2*, CS-*cp4 epsps*, and T-*TubA*) and one *lox*P site were absent. These results were fully consistent with the NGS dataset.

To examine the T-DNA insertion site, PCR primers flanking the insertion site were used to amplify genomic DNA from MON95275 and from LH244. Comparing the products from MON95275 and LH244 identified a 746 base deletion of the corn genomic DNA that occurred during T-DNA integration, as well as a 6 base insertion in the 3' flanking sequence. All other flanking sequences in MON95275 were identical to those in LH244. Such changes during T-DNA insertion are common during *Agrobacterium*-mediated plant transformation due to double-stranded break repair mechanisms (Salomon and Puchta 1998; Anderson et al. 2016) and would not affect the expression of the *DvSnf7.1, mpp75Aa1.1,* or *vbp4Da2* cassettes.

3.4.4   Stability of the genetic changes in corn line MON95275

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

Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

3.4.4.1 Genetic stability

NGS was used to show the genetic stability of the inserted DNA in MON95275 (F4) by evaluating seed-derived DNA from four additional breeding generations of MON95275 (F4F1, F5, F5F1, F6). Control genomic DNA was isolated from (1) the non-GM parental line LH244, which shares similar background genetics to the F4, F5 and F6 generations and (2) the non-GM hybrid line LH244 × HCL617 with similar background genetics to the F4F1 and F5F1 hybrids.

The analysis showed that the two insert-flank junction sequences present in the F4 generation (section 3.4.1) were identical in each of the four additional generations. No other junction sequences were present. These results confirm that a single T-DNA insert is stably integrated in MON95275.

3.4.4.2 Pattern of inheritance

Mendelian inheritance

Since the inserted T-DNA resides at a single locus within the MON95275 genome, it would be expected to be inherited according to Mendelian principles. To confirm this, three generations of MON95275 (F4F2, F4F3, and F4F4; see Figure 4) were tested for the presence of T-DNA using a Real Time TaqMan® PCR assay, and a Pearson's chi-square (χ^2) analysis was conducted to compare the observed and expected segregation ratios across these generations.

According to Mendelian inheritance principles, the predicted segregation ratio in all generations was 1:2:1 (homozygous positive: hemizygous positive: homozygous negative). The χ^2 critical value to reject the hypothesis of this ratio at a 5% level of significance was 5.99 (Strickberger 1976). The results of the χ^2 analyses on all three generations tested gave χ^2 values < 5.99 (Table 3), indicating there were no significant differences between the observed and expected segregation ratios in any of the generations. These data support the conclusion that the inserted DNA is present at a single locus in the MON95275 genome and is inherited predictably according to Mendelian inheritance principles.
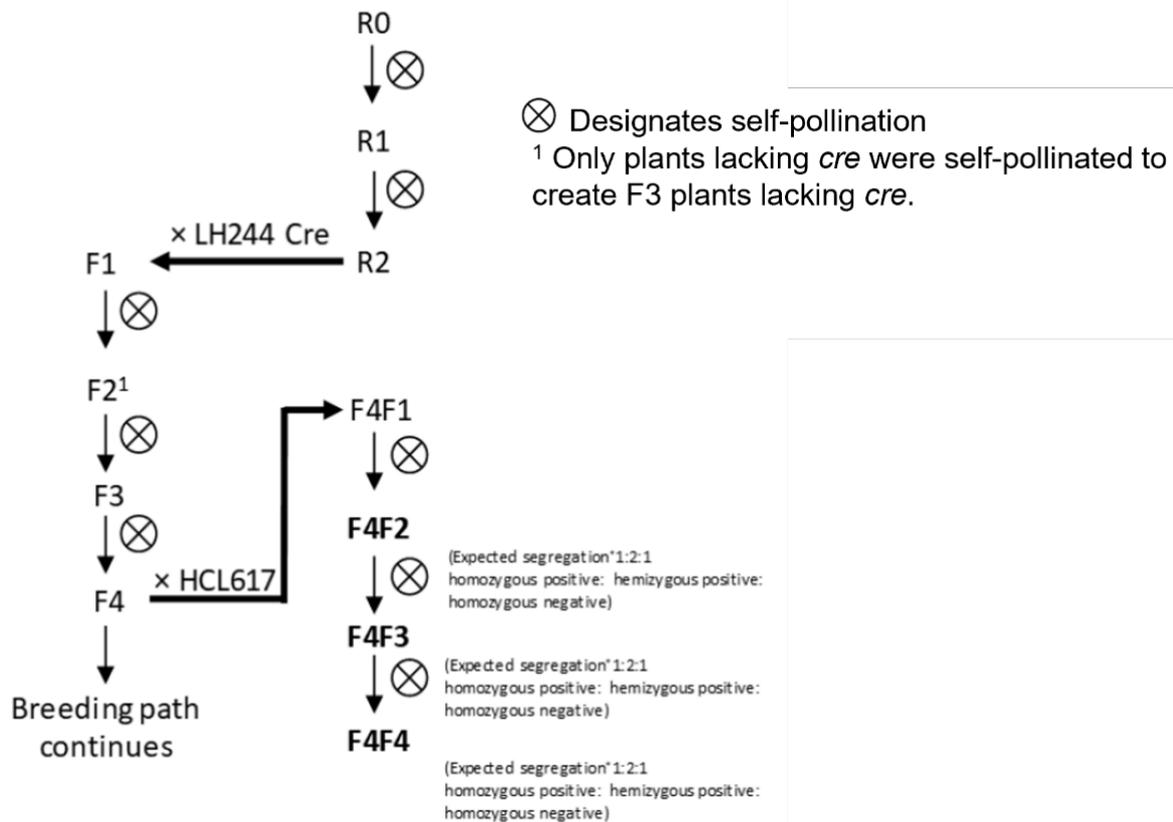
Figure 4. *Breeding path used to assess the inheritance and genetic stability of MON95275. F4 MON95275 was crossed with the proprietary elite inbred line HCL617 to produce a F4F1 hemizygous seed. Self-crossing this line is expected to segregate at a 1:2:1 ratio (homozygous positive: hemizygous positive: homozygous negative). Continual self-crossing of hemizygous progeny is expected to result in the same ratios. Bolded text indicates generations whose segregation data was used in the χ^2 analysis.*

Table 3: Segregation results in three generations of MON95275

Generation	Expected segregation ratio	Observed number of plants (expected number)				Statistical analysis	
		Homozygous Positive	Hemizygous positive	Homozygous Negative	Total	χ^2	*P value*
F4F2	1:2:1	87 (87.5)	169 (175)	94 (87.5)	350	0.69	0.708
F4F3	1:2:1	63 (73.25)	152 (146.5)	78 (73.25)	293	1.95	0.377
F4F4	1:2:1	75 (75.5)	147 (151)	80 (75.5)	302	0.38	0.828

Expression of phenotype over several generations

The expression of the Mpp75Aa1.1 and Vpb4Da2 proteins in five generations of MON95275 (F4, F4F1,F5, F5F1 and F6; see Figure 2) was examined. Western blot analysis was conducted on grain tissue from each generation, with corn lines LH244 and LH244 × HCL617 used as negative controls, and *E. coli*-produced Mpp75Aa1.1 and Vpb4Da2 proteins (see Section 4.1.1 and 4.1.2) used as positive controls. In all five breeding generations, both Mpp75Aa1.1 and Vpb4Da2 migrated indistinguishably from the corresponding positive controls. Neither protein was detected in the tissue from the conventional controls. These data support the conclusion that the Mpp75Aa1.1 and Vpb4Da2 proteins are stably expressed over several generations.

3.4.5   Open reading frame analysis

A bioinformatic analysis of the MON95275 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel open reading frames (ORFs) had been created in MON95275 as a result of T-DNA insertion, and whether any putative peptides or polypeptides present in the insert have the potential for allergenicity or toxicity.

Sequences spanning the 5' and 3' insert-flank junctions of MON95275 were translated *in silico* from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames[9]. Similarly, the entire MON95275 insert DNA was translated in all six reading frames. A total of 10 ORFs were identified that corresponded to putative peptides of eight amino acids or greater in length from the insert-flank junction sequences. Along with the 6 putative polypeptides translated from the insert sequence, these were investigated further to determine whether their amino acid sequences showed similarity with known allergen and toxin peptide sequences in established databases.

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

3.4.5.1 Bioinformatic analysis for potential allergenicity

The putative peptides present in the 5' and 3' insert-flank junction sequences and the amino acid sequences encoded by all six reading frames in the MON95275 insert DNA were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource (COMPARE[10]) database, from the Health and Environmental Science Institute. At the date of the search, there were 2,348 sequences in the allergen database (AD_2021). Sequences were also compared to the GenBank all protein database (PRT_2021), downloaded from the National Centre for Biotechnology Information (NCBI[11]), which contained 139,450,651 sequences at the date of download.

Three types of analyses were performed for this comparison:

(a) Full length sequence search – a FASTA alignment using a BLOSUM50 scoring matrix, which identifies blocks of residues with at least 50% sequence identity. Only matches with E-scores of $\leq 1 \times 10^{-5}$ were considered.

(b) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids to the database entries. Only matches of greater than 35% similarity over ≥ 80 amino acids were considered.

(c) 8-mer exact match search – an in-house algorithm was used to identify whether an 8 amino acid peptide match existed between the query sequences and sequences within the allergen database. Only matches of 100% similarity over 8 amino acids were considered.

The alignment of the 10 putative peptides present in the 5' and 3' insert-flank junctions with database sequences did not identify any matches. Some matches were identified when the putative peptides encoded by the six reading frames in the MON95275 insert DNA were aligned with the allergen (AD_2021) and all protein database (PRT_2021). However, these matches either contained numerous stop codons, or, in the case of the 8-mer matches, were almost entirely comprised of either serine or leucine, indicating these matches were likely the result of low sequence complexity, not structural homology. The results of this analysis support the conclusion that there were no matches of significance or concern.

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

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

[11] https://www.ncbi.nlm.nih.gov/protein/

3.4.5.2 Bioinformatic analysis for potential toxicity

The putative peptides encoded by the junction and insert sequences were also compared *in silico* to a toxin protein database (TOX_2021). This database is a subset of sequences derived from the UniProtKB/Swiss-Prot protein databases, curated to remove likely non-toxin proteins, and contained 7,870 sequences at the date of analysis. A FASTA algorithm was used with a BLOSUM50 scoring matrix and the E-value threshold conservatively set to 1×10^{-5}. No matches were found between the 10 putative junction polypeptides and any known protein toxins.

Two of the six *in silico* translated reading frames present in the MON95275 insert DNA each yielded a single alignment. These alignments corresponded to the expected translation products Mpp75Aa1.1 and Vpb4Da2, which both possess broad homology to exotoxin-B β-pore forming protein family sequences in the TOX_2021 database. However, neither Mpp75Aa1.1 nor Vpb4Da2 possess homology with the receptor-binding domains of these proteins, and are unlikely to represent mammalian toxins (see sections 4.1.1.3 and 4.1.2.3 for further detail). Therefore, these matches are of no significance or concern.

3.5 Expression of RNA transcripts

Northern blotting of total RNA isolated from various MON95275 tissues showed that DvSnf7.1 was detected in root and leaf tissue but not in grain or pollen. This result is similar to MON87411 (FSANZ, 2015), where the amount of DvSnf7 dsRNA present in the grain of MON87411 was extremely low (0.104 ng/g dw). The only difference between the DvSnf7.1 transcript in MON95275 and the DvSnf7 transcript in MON87411 is the 5'UTR, which has been modified in MON95275 for improved expression *in planta*.

In addition to the expected DvSnf7.1 band in MON95275 leaf tissue, three read-through transcripts of >6 kb in size were detected via Northern blotting and sequenced. The positioning of any start codons in these transcripts downstream of the inverted repeat region means they would be inaccessible to ribosome assembly (Kozak 1989; Hinnebusch 2011). In addition, after degradation of the inverted repeat RNA by endogenous RNases, any remaining downstream ssRNA sequences would lack a 5' cap, which would prevent ribosome binding. Furthermore, these capless sequences would likely be subject to RNA degradation themselves (Labno et al. 2016). Therefore, there is a low probability that these read-through transcripts would be able to initiate translation. Nonetheless, all putative peptides were screened against the allergen (AD_2021) and toxin (TOX_2021) databases, and did not yield any meaningful alignments. Taken together, these read-through transcripts are not of significance or concern.

3.6 Conclusion

The data provided by the applicant showed that a single integration of T-DNA has occurred at a specific locus in the MON96275 genome. DNA sequencing confirmed that the *DvSnf7.1*, *mpp75Aa1.1* and *vpb4Da2* cassettes were inserted with the expected sequences and organisation. The *cp4 epsps* selectable marker cassette, which was inserted as part of the initial T-DNA transformation, is absent in MON95275 as a result of excision by Cre/*lox* recombination. No plasmid backbone sequences, including antibiotic resistance genes, are evident in MON95275.

The inserted DNA is stably inherited and expressed across several breeding generations of MON95275. Northern blotting analysis showed that DvSnf7.1 RNA was not detected in grain and pollen of MON95275. None of the new ORFs created by the modification, or read-through transcripts from the *DvSnf7.1* cassette, raised any allergenicity or toxicity concerns.

4 Characterisation and safety assessment of novel substances

Three novel substances are expressed in MON95275: the Mpp75Aa1.1 and Vpb4Da2 insecticidal proteins which provide protection against corn rootworm; and the DvSnf7 dsRNA molecule which mediates RNAi-silencing of the corn rootworm *DvSnf7* gene. In considering the safety of newly expressed substances it is important to note that a large and diverse range of proteins and dsRNAs are ingested as part of the normal human diet without any adverse effects.

4.1 Novel Proteins

Only a small number of dietary proteins have the potential to impair health, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutrient or allergenic effects.

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

Two novel proteins are expressed in MON95275, Mpp75Aa1.1 and Vpb4Da2. These proteins were previously designated Cry75Aa1.1 and Vip4Da2, respectively, but were recently reclassified based on a revised nomenclature system developed for insecticidal proteins (Crickmore et al. 2021). Despite their reclassification, both proteins have the same general mode of action as members of the crystal (Cry) family of pore-forming proteins produced by *B. thuringiensis.* Upon ingestion by the target pest, alkaline conditions and proteases in the insect midgut cause proteolytic cleavage of the protein's protoxin domain and activation of the insecticidal toxin. The activated protein functions by binding to a highly specific glycoprotein receptor on the surface of midgut epithelial cells, aggregating and forming pores in the cell membrane (Schnepf et al. 1998). This leads to loss of cell integrity in the midgut, leading to growth inhibition and insect death.

4.1.1 Mpp75Aa1.1

The Mpp75Aa1.1 protein is a 295 amino acid, ~34 kDa protein which is a member of the ETX_MTX2 subfamily of β-pore forming proteins (β-PFPs). Like other members of the ETX_MTX2 family, Mpp75Aa1.1 is composed of three domains: Domain I acts as the receptor binding region, and Domains II and III are involved in pore formation. Domains II and III are structurally similar across members of the ETX_MTX2 family, while the receptor-binding domain is more structurally diverse (Moar et al. 2017).

For activity against WCR, the carboxyl-terminus of Mpp75Aa1.1 is first processed by WCR midgut proteases, after which it forms an active oligomer. The active oligomer binds the midgut microvilli resulting in damage to the insect gut lumen (Kouadio et al. 2021a). At least three amino acids in the receptor binding domain appear to be responsible for the insecticidal specificity of Mpp75Aa1.1 (Kouadio et al. 2021a), which is active against WCR but not against other coleopteran species (Bowen et al. 2021). Mpp75Aa1.1 also exhibits activity toward WCR larvae that are resistant to certain Cry family proteins previously employed against WCR, suggesting a distinct receptor specificity (Bowen et al. 2021).

4.1.1.1 Characterisation of Mpp75Aa1.1 expressed in MON95275 and equivalence to a bacterially-produced form

The equivalence of the MON95275- and *E.coli*-derived Mpp75Aa1.1 proteins must be established before the safety data generated using *E. coli*-derived Mpp75Aa1.1 can be applied to MON95275-derived Mpp75Aa1.1.

Plant-derived Mpp75Aa1.1 protein was purified from MON95275 grain using a combination of anion-exchange and immunoaffinity chromatography. The purified fractions containing Mpp75Aa1.1 were identified by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot, and subsequently concentrated. *E.coli*-derived Mpp75Aa1.1 was generated from *E. coli* containing a plasmid that expresses Mpp75Aa1.1.

In order to confirm the identity and equivalence of the MON95275- and *E. coli* derived Mpp75Aa1.1, a series of analytical tests were employed, the results of which are summarised below.

Molecular weight. Samples of purified MON95275- and *E. coli*-derived Mpp75Aa1.1 were subjected to SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. The average purity of the MON95275-derived Mpp75Aa1.1, as judged by SDS-PAGE, was determined to be 70%. The average apparent molecular weights of the MON95275- and *E. coli*-derived Mpp75Aa1.1 were 35.2 and 34.0 kDa, respectively. This is within the acceptable limits for molecular weight equivalence.

Immunoreactivity. Western blot analysis with a Mpp75Aa1.1-specific antibody showed that the protein being expressed in MON95275 and *E. coli* was indeed Mpp75Aa1.1, and that they have equivalent immunoreactivity.

N-terminal sequencing. MON95275- and *E. coli*-derived Mpp75Aa1.1 were digested with trypsin and analysed by mass spectrometry (LC-MS/MS). The N-terminal sequence of both proteins matched the expected sequence based on the *mpp75Aa1.1* gene, though in both cases the N-terminal methionine had been cleaved *in vivo*, a common process in many organisms (Bradshaw et al. 1998).

Peptide mapping. MON95275-derived and *E. coli*-derived Mpp75Aa1.1 were digested with trypsin and analysed by LC-MS/MS. For MON95275-derived Mpp75Aa1.1, 41 unique peptides were identified, covering 96% of the expected protein sequence (283 of 295 amino acids). For *E. coli*-derived Mpp75Aa1.1, 61 unique peptides were identified, covering 99.7% of the expected protein sequence (294 of 295 amino acids), along with a small population of sequences corresponding to the protein with an intact N-terminal methionine. These results further confirm that the protein being expressed in MON95275 and *E. coli* is Mpp75Aa1.1.

Glycosylation analysis. An SDS-PAGE and glycoprotein detection procedure involving enhanced chemiluminescence (ECL) showed that Mpp75Aa1.1 from both MON95275 and *E. coli* was equivalent and that neither is glycosylated. The positive control protein (transferrin) showed a band indicative of glycosylation.

Functional activity. The biological activity of MON95275- and *E. coli*-derived Mpp75Aa1.1 was evaluated in a 7-day insect bioassay. In this assay, WCR larvae fed a diet containing MON95275- and *E. coli*-derived Mpp75Aa1.1 showed a mean EC_{50} value[12] of 9.4 µg protein/ml diet and 6.8 µg protein/ml diet, respectively. These EC_{50} values are within

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[12] The concentration of protein causing a 50% growth inhibition compared to the control treatment.

acceptance limits for equivalence.

The results outlined in this section demonstrated that *E.coli*-derived Mpp75Aa1.1 is structurally, biochemically and functionally equivalent to MON95275-derived Mpp75Aa1.1. It can be concluded that *E. coli*-derived Mpp75Aa1.1 is a suitable surrogate for use in the safety studies described in Section 4.1.1.3.

4.1.1.2 *Expression of Mpp75Aa1.1 in MON95275 tissue*

For analysis of the expression levels of Mpp75Aa1.1 protein in MON95275, tissues were collected from five field-trial sites in representative corn-producing regions of the United States during the 2019 growing season[13]. Tissues were collected at varying stages of growth (see Figure 5 for a summary of corn growth stages). Mpp75Aa1.1 was extracted from tissues using standard methods and expression levels were quantified in each tissue using a quantitative enzyme-linked immunosorbent assay (ELISA). *E. coli*-derived Mpp75Aa1.1 protein, as described in Section 4.1.1.1, was used as an analytical reference for plant-derived Mpp75Aa1.1. For each tissue analysed, four samples were processed from each of the five field-trial sites.
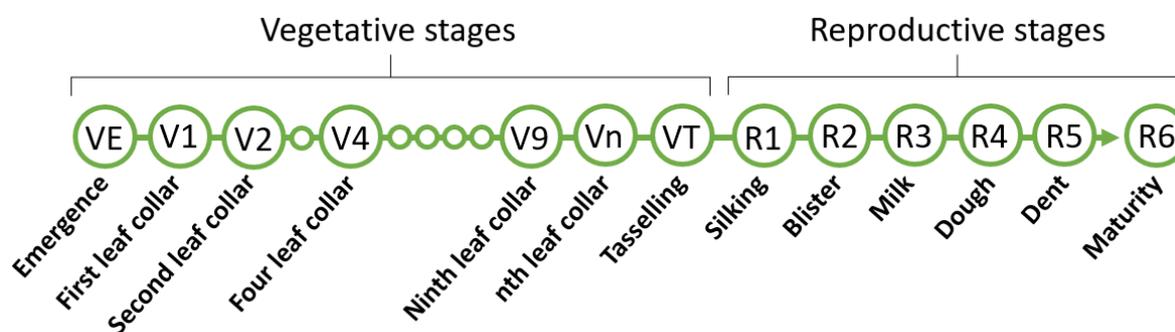
Figure 5. *Stages of corn growth. Grain is harvested at maturity (R6).*

Results from the ELISA show that the highest Mpp75Aa1.1 expression in MON95275 was in leaf tissue in the early V2-V4 vegetative stage (Table 4). The lowest level of expression was in pollen at the VT-R1 stage, which was below the limit of quantitation for the assay (0.125 µg/g dry weight). Grain at maturity (R6) also contained a low level of Mpp75Aa1.1 (1.3 µg/g dw).

Table 4: Mpp75Aa1.1 concentrations (µg/g dw[1]) in MON95275 tissues

Tissue	Growth Stage[2]	Mean	SE[3]	Range
Leaf	V2-V4	100	7.0	43 – 200
Root	V2-V4	35	4.3	11 – 84
Pollen	VT-R1	<LOQ[4]	N/A[5]	N/A
Forage	R5	16	0.76	12 – 25
Grain	R6	1.3	0.086	0.67 – 1.9

1. dw - dry weight 2. Growth Stage abbreviations – see Figure 5 3. SE – standard error 4. LOQ – limit of quantitation (0.125 µg/g dw) 5. N/A – not applicable

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[13] Field sites for testing protein expression levels were in the following United States states – Iowa, Illinois (two sites), Indiana and Missouri

4.1.1.3 Safety of the introduced Mpp75Aa1.1

Data were provided to assess the potential toxicity and allergenicity of Mpp75Aa1.1.

Bioinformatic analyses of Mpp75Aa1.1

Bioinformatic analyses, as described in Section 3.4.5.1, were performed to compare the Mpp75Aa1.1 amino acid sequence to known allergenic proteins in the COMPARE allergen database (AD_2021). The search did not identify any known allergens with homology to Mpp75Aa1.1. No alignments had an E-score of $\leq 1 \times 10^{-5}$ or met or exceeded the threshold of greater than 35% similarity over ≥ 80 amino acids, and no eight amino acid peptide matches were shared between the Mpp75Aa1.1 sequence and proteins in the allergen database.

To assess the similarity of Mpp75Aa1.1 to known toxins, an *in silico* analysis was conducted to compare the Mpp75Aa1.1 amino acid sequence to proteins identified as "toxins" in the TOX_2021 database, as described in Section 3.4.5.2. This query returned a single alignment with an E-score of $\leq 1 \times 10^{-5}$, to Q02307, an epsilon-toxin type B (ETX) from *Clostridium perfringens*. This alignment, which displayed 25.9% identity and a E-score of 1.1×10^{-22}, is expected as Mpp75Aa1.1 is a member of the ETX-MTX2 sub-family of bacterial β-pore forming proteins (Bowen et al. 2020).

ETX-MTX2 family members contain three domains, with the structurally-diverse Domain I being responsible for receptor binding and toxin specificity (Moar et al. 2017). The alignment between Mpp75Aa1.1 and Q02307 reveals that the similarity in Domain I is lower than for Domains II and III, and, importantly, that only two of the six residues in Domain I identified as critical for mammalian toxicity in Q02307 (Moar et al. 2017) are shared by Mpp75Aa1.1. Furthermore, comparison of the Mpp75Aa1.1 and epsilon toxin crystal structures shows that there are significant structural differences between their receptor-binding domains (Kouadio et al. 2021a). This lack of sequence and structural identity in the receptor-binding domain indicates that Mpp75Aa1.1 is unlikely to be a mammalian toxin.

As expected, comparing the Mpp75Aa1.1 sequence to all proteins in the NCBI database (PRT_2021) self-identified Mpp75Aa1.1, and also returned several alignments to other insecticidal proteins from the ETX_MTX2 family. None of these proteins are mammalian toxins, and as such these alignments do not represent a safety concern.

Susceptibility of Mpp75Aa1.1 to digestion with pepsin and pancreatin

E. coli-produced Mpp75Aa1.1 (test protein) was mixed with pepsin at a concentration of 10U enzyme/μg protein, then incubated for 0-60 min in a 37.4°C water bath. Reactions occurred under acidic conditions in simulated gastric fluid (Thomas et al. 2004). A control mixture lacking pepsin, and a separate control lacking Mpp75Aa1.1, were also incubated for 60 min and analysed at 0 min and 60 min.

The extent of digestion was visualised by Brilliant Blue G-colloidal stained SDS-PAGE and Western blotting with an anti-Mpp75a1.1 antibody. Concurrently, serial dilutions of the reaction mixture (test protein plus pepsin) without incubation (0 min) were subjected to SDS-PAGE, followed by either gel staining or Western blotting, to estimate the limit of detection (LOD) for Mpp75Aa1.1. For the Brilliant Blue-stained gel, the LOD was estimated to be ~1.6 ng and 1 μg of test protein was loaded per lane to assess digestibility. In the Western blotting experiments, the LOD was estimated to be ~0.031 ng and 10 ng test protein was loaded per lane to assess digestibility.

Visual inspection of both the stained gel and the Western blot showed that by 0.5 min of incubation, the amount of intact Mpp75Aa1.1 remaining in the reaction mix was below the

LOD. Based on the LOD, it was calculated that >99.8% of the intact Mpp75Aa1.1 was digested within 0.5 min when analysed by SDS-PAGE and >99.7% when analysed by Western blot. A single peptide fragment of ~4 kDa was observed in the stained SDS-PAGE in the 2 min sample, but not at any of the later time intervals. No smaller peptides were detected in the Western blot at any time beyond 0.5 min. Mpp75Aa1.1 in the control mixture lacking pepsin remained intact after 60 min of incubation, indicating that the rapid loss of Mpp75Aa1.1 protein in the reaction mixes is due to proteolytic digestion of Mpp75Aa1.1 by pepsin and not due to instability of the protein while in the simulated gastric fluid for 60 min.

To assess the susceptibility of Mpp75Aa1.1 to pancreatin[14] digestion, *E. coli*-derived Mpp75Aa1.1 was incubated with pancreatin (~55 µg enzyme/µg protein) at 37.1°C for 0-24 h, in a simulated intestinal fluid system at a neutral pH range. A control mixture lacking pancreatin, and a separate control lacking Mpp75Aa1.1, were also incubated for 24 h and analysed at 0 min and 24 h. The extent of digestion was visualised by Western blot. A serial dilution of the reaction mixture (test protein plus enzyme) without incubation was used to determine the LOD for the protein, which was approximately 0.013 ng. For the digestibility analysis, ~10 ng of the test protein was loaded per lane.

Visual inspection of the Western blot showed that after 15 min of incubation, the level of intact Mpp75Aa1.1 remaining in the reaction mix was below the LOD. Based on the LOD, it was calculated that >99.7% of the intact Mpp75Aa1.1 protein was degraded within 15 min. Several faint bands of <30 kDa were present throughout the period of digestion, corresponding to fragments of Mpp75Aa1.1. However, the sensitivity of the blot means that these fragments represent very small quantities. In the human digestive system, gastric digestion (pepsin) occurs before intestinal digestion (pancreatin). Such fragments would not be expected to be present if the protein had been exposed to pepsin prior to incubation with pancreatin, based on the rapid and complete digestion of Mpp75Aa1.1 by pepsin described above. Mpp75Aa1.1 in the control mixture lacking pancreatin remained intact after 24 h of incubation, indicating that the degradation of Mpp75Aa1.1 in the reaction mixes is indeed due to proteolytic digestion of Mpp75Aa1.1 by pancreatin.

Taken together, these results indicate that Mpp75Aa1.1 will be fully degraded by gastric and intestinal enzymes in the human digestive system.

Structural stability and bioactivity of Mpp75Aa1.1 after exposure to heat

E. coli-produced Mpp75Aa1.1 was heated for 15 or 30 min at temperatures ranging from 25-95°C. A control sample was kept on wet ice (~0°C). Aliquots of the control and heated protein samples were subjected to SDS-PAGE and stained with Brilliant Blue G-Colloidal stain, with the extent of protein degradation assessed qualitatively. No visible degradation or decrease in band intensity was observed for Mpp75Aa1.1 in the control, 25, 37, or 55°C treated samples at either 15 or 30 min. At 75°C, some smaller molecular weight degradation products were present after 15 min, and a higher intensity of these degradation products were observed after 30 min. Some high molecular weight aggregation products were observed at 37, 55 and 75°C, which decreased in intensity with increasing temperature. The samples treated at 95°C for 15 and 30 min showed a marked loss of Mpp75Aa1.1 band intensity and presence of degradation products, with the most prominent degradation evident after 30 min. These data indicate that Mpp75Aa1.1 is heat labile at temperatures greater than 75°C.

To assess the functional activity of Mpp75Aa1.1 after heating, serial dilutions of the heated protein samples were tested in a bioassay using WCR larvae similar to that described in Section 4.1.1.1. Control insects were fed a diet containing unheated Mpp75Aa1.1 which had

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[14] Pancreatin is a mixture of proteolytic enzymes

been maintained at ~0°C. The bioactivity for each sample was measured as a 7-day EC_{50} value, which is the Mpp75Aa1.1 concentration that results in a 50% growth inhibition relative to control insects. At heating temperatures of 25 or 37°C for either 15 or 30 min, the activity of Mpp75Aa1.1 remained similar to that of the unheated Mpp75Aa1.1 (Table 5). At 55°C, protein activity was maintained after 15 min, but for the 30 min heat treatment activity was reduced to the extent that an EC_{50} value could not be established (<50% growth inhibition at the highest protein concentration tested). Similarly, EC_{50} values could not be calculated for the samples heated to 75 or 95°C for either 15 or 30 min, indicating that heating to temperatures of ≥75°C or above, or at 55°C for 30 min, effectively denatures the Mpp75Aa1.1 protein and it loses its functional activity.

Table 5: Bioactivity of heat-treated Mpp75Aa1.1 in a diet fed to insect larvae

Temperature	15 min heat treatment		30 min heat treatment	
	EC_{50} (µg Mpp75Aa1.1/ml diet)	95% CI[b] (µg Mpp75Aa1.1/ml diet)	EC_{50} (µg Mpp75Aa1.1/ml diet)	95% CI[b] (µg Mpp75Aa1.1/ml diet)
0°C (control)	7.3	4.2 – 13	7.3	4.2 – 13
25°C	15	6.8 – 34	12	6.2 – 23
37°C	12	8.1 – 18	7.1	4.8 – 11
55°C	15	8.4 – 28	N/A[a]	N/A
75°C	N/A[a]	N/A	N/A[a]	N/A
95°C	N/A[a]	N/A	N/A[a]	N/A

a. The EC_{50} could not be calculated as <50% growth inhibition was observed at the highest protein concentration tested.
b. Confidence limits

4.1.2   Vpb4Da2

The *vpb4Da2* gene in MON95275 encodes the full length native Vpb4Da2 protein from *B. thuringiensis*. This protein is comprised of 937 amino acids and has a molecular weight of ~104 kDa.

The Vpb4Da2 protein is a member of the bacterial exotoxin-B class of β-PFPs and is highly selective in its insecticidal activity against WCR (Yin et al. 2020). Vpb4Da2 is composed of 6 structural domains. Domains I-III are structurally homologous to domains found in a wide variety of bacterial and eukaryotic proteins, known as PA14 domains (Rigden et al. 2004). The C-terminal Domains IV-VI adopt specific carbohydrate-binding architectures and are required for activity against WCR. After processing by WCR midgut proteases, these domains bind to putative epithelial receptors in the midgut resulting in disruption to the gut lumen and insect death (Kouadio et al. 2021b).

4.1.2.1 *Characterisation of Vpb4Da2 expressed in MON95275 and equivalence to a bacterially-produced form*

The equivalence of the MON95275- and *E.coli*-derived Vpb4Da2 proteins must be established before the safety data generated using *E. coli*-derived Vpb4Da2 can be applied to MON95275-derived Vpb4Da2.

Plant-derived Vpb4Da2 protein was purified from MON95275 grain using a combination of anion-exchange and immunoaffinity chromatography. The purified fractions containing Vpb4Da2 were identified by SDS-PAGE and Western blot, and subsequently concentrated. *E.coli*-derived Vpb4Da2 was generated from *E. coli* containing a plasmid that expresses Vpb4Da2.

In order to confirm the identity and equivalence of the MON95275- and *E. coli* derived Vpb4Da2, a series of analytical tests were employed, the results of which are summarised below.

Molecular weight. Samples of purified MON95275- and *E. coli*-derived Vpb4Da2 were subjected to SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. The average purity of the MON95275-derived Mpp75Aa1.1, as judged by SDS-PAGE, was determined to be 26%. The average apparent molecular weights of the MON95275- and *E. coli*-derived Vpb4Da2 were 104.9 and 103.8 kDa, respectively, which is within the acceptable limits for molecular weight equivalence.

Immunoreactivity. Western blot analysis with a Vpb4Da2-specific antibody showed that the protein being expressed in MON95275 and *E. coli* was indeed Vpb4Da2, and that they have equivalent immunoreactivity.

N-terminal sequencing. MON95275-derived and *E. coli*-derived Vpb4Da2 were digested with trypsin and analysed by mass spectrometry (LC-MS/MS). The N-terminal sequence of both proteins matched the expected sequence based on the *vpb4Da2* gene.

Peptide mapping. MON95275-derived and *E. coli*-derived Vpb4Da2 were digested with trypsin and analysed by LC-MS/MS. For MON95275-derived Vpb4Da2, 121 unique peptides were identified, covering 98% of the expected protein sequence (914 of 937 amino acids). For *E. coli*-derived Vpb4Da2, 182 unique peptides were identified, covering 99% of the expected protein sequence (929 of 937 amino acids). These results further confirm that the protein being expressed in MON95275 and *E. coli* is Vpb4Da2.

Glycosylation analysis. An SDS-PAGE and glycoprotein detection procedure involving enhanced chemiluminescence (ECL) showed that Vpb4Da2 from both MON95275 and *E. coli* was equivalent and that neither is glycosylated. The positive control protein (transferrin) showed a band indicative of glycosylation.

Functional activity. The biological activity of MON95275- and *E. coli*-derived Vpb4Da2 was evaluated in a 7-day insect bioassay. In this assay, WCR larvae fed diet containing MON95275- and *E. coli*-derived Vpb4Da2 showed a mean EC_{50} value of 12.3 µg protein/ml diet and 6.1 µg protein/ml diet, respectively. These EC_{50} values are within acceptance limits for equivalence.

The results outlined in this section demonstrated that *E.coli*-derived Vpb4Da2 is structurally, biochemically and functionally equivalent to MON95275-derived Vpb4Da2. It can be concluded that *E. coli*-derived Vpb4Da2 is a suitable surrogate for use in the safety assessment experiments described in Section 4.1.2.3.

4.1.2.2 Expression of Vpb4Da2 in MON95275 tissue

The levels of Vpb4Da2 expression in plant tissues was determined in the same ELISA study described in Section 4.1.1.2, using *E. coli*-produced Vpb4Da2 as an analytical reference for plant-derived Vpb4Da2.

Results from the ELISA show that the highest Vpb4Da2 expression in MON95275 was in leaf

tissue in the early V2-V4 vegetative stage (Table 6). The lowest level of expression was in pollen at the VT-R1 stage, which was below the limit of quantitation for the assay (0.157 µg/g dry weight). Grain at maturity (R6) also contained a very low level of Vpb4Da2 (1.2 µg/g dw).

Table 6: Vpb4Da2 concentrations (µg/g dw[1]) in MON95275 tissues

Tissue	Growth Stage[2]	Mean	SE[3]	Range
Leaf	V2-V4	39	1.8	19 – 51
Root	V2-V4	14	1.3	3.4 – 26
Pollen	VT-R1	<LOQ[4]	N/A[5]	N/A
Forage	R5	3.3	0.13	2.5 – 4.8
Grain	R6	1.2	0.086	0.42 – 1.9

[1.] dw - dry weight [2.] Growth Stage abbreviations – see Figure 5 [3.] SE – standard error [4.] LOQ – limit of quantitation (0.157 µg/g dw) [5.] N/A – not applicable

4.1.2.3 Safety of the introduced Vpb4Da2

Bioinformatic analyses of Vpb4Da2

Bioinformatic analyses, as described in Section 3.4.5.1, were performed to compare the Vpb4Da2 amino acid sequence to known allergenic proteins in the COMPARE allergen database (AD_2021). The search did not identify any known allergens with homology to Vpb4Da2. No alignments had an E-score of $\leq 1 \times 10^{-5}$ or met or exceeded the threshold of greater than 35% similarity over ≥ 80 amino acids, and no eight amino acid peptide matches were shared between the Vpb4Da2 sequence and proteins in the allergen database.

To assess the similarity of Vpb4Da2 to known toxins, an *in silico* analysis was conducted to compare the Vpb4Da2 amino acid sequence to proteins identified as "toxins" in the TOX_2021 database, as described in Section 3.4.5.2. This query returned a single alignment with an E-score of $\leq 1 \times 10^{-5}$, to the Protective Antigen (PA) P13423 from *Bacillus anthracis*. This alignment displayed 32.7% identity over 626 amino acids and an E-score of 7.2×10^{-58}.

Domains I-III of Vpb4Da2 possess a domain architecture which is conserved across the bacterial exotoxin-B β-PFP family, of which PA is a member (Yin et al. 2020). The majority of the sequence homology in the Vpb4Da2 alignment with P13423 was found in domains I-III, which are responsible for oligomerisation and membrane insertion. The receptor-binding Domain IV of P13423 is more variable and is responsible for specificity (Yin et al. 2020). This domain showed minimal sequence homology with Vpb4Da2, with only two of 140 amino acids shared between P13423 and Vpb4Da2 in this region. Due to the lack of homology in the receptor-binding domain, Vpb4Da2 is unlikely to be a mammalian toxin.

As expected, comparing the Vpb4Da2 sequence to all proteins in the NCBI database (PRT_2021) self-identified Vpb4Da2, and also returned several alignments to other insecticidal proteins from *B. thuringiensis*. None of these proteins are mammalian toxins, and as such these alignments do not represent a safety concern.

Susceptibility of Vpb4Da2 to digestion with pepsin and pancreatin

Vpb4Da2 (test protein) was mixed with pepsin at a concentration of 10U enzyme/µg protein, then incubated for 0-60 min in a 38.4°C water bath. Reactions occurred under acidic conditions in simulated gastric fluid (Thomas et al. 2004). A control mixture lacking pepsin, and a separate control lacking Vpb4Da2, were also incubated for 60 min and analysed at 0 min and 60 min.

The extent of digestion was visualised by Brilliant Blue G-colloidal stained SDS-PAGE and

Western blotting with an anti-Vpb4Da2 antibody. Concurrently, serial dilutions of the reaction mixture (test protein plus pepsin) without incubation (0 min) were subjected to SDS-PAGE, followed by either gel staining or Western blotting, to estimate the limit of detection (LOD) for Vpb4Da2. For the Brilliant Blue-stained gel, the LOD was estimated to be ~1.6 ng, and 1 μg of test protein was loaded per lane to assess digestibility. In the Western blotting experiments, the LOD was estimated to be ~0.063 ng, and ~5 ng test protein was loaded per lane to assess digestibility.

Visual inspection of both the stained gel and the Western blot showed that after 0.5 min of incubation, the amount of intact Vpb4Da2 remaining in the reaction mix was below the LOD. Based on the LOD, it was calculated that >99.8% of the intact Vpb4Da2was digested within 0.5 min when analysed by SDS-PAGE and >98.7% when analysed by Western blot. Peptide fragments between 2.5 and ~6 kDa were observed in the stained SDS-PAGE for the first 5 mins of pepsin digestion, but not at any of the later time intervals. No smaller peptides were detected in the Western blot at any time beyond 0.5 min. Vpb4Da2 in the control mixture lacking pepsin remained intact after 60 min of incubation, indicating that the rapid loss of Vpb4Da2 protein in the reaction mixes is due to proteolytic digestion of Vpb4Da2 by pepsin and not due to instability of the protein while in the simulated gastric fluid for 60 min.

To assess the susceptibility of Vpb4Da2 to pancreatin digestion, *E. coli*-derived Vpb4Da2 was incubated with pancreatin (~55 μg enzyme/μg protein) at 38.2°C for 0-24 h, in a simulated intestinal fluid system at a neutral pH range. A control mixture lacking pancreatin, and a separate control lacking Vpb4Da2, were also incubated for 24 h and analysed at 0 min and 24 h. The extent of digestion was visualised by Western blot. A serial dilution of the reaction mixture (test protein plus enzyme) without incubation was used to determine the LOD for the protein, which was approximately 0.063 ng. For the digestibility analysis, ~5 ng of the test protein was loaded per lane.

Visual inspection of the Western blot showed that after 5 min of incubation, the level of intact Vpb4Da2 remaining in the reaction mix was below the LOD. Based on the LOD, it was calculated that >98.7% of the intact Vpb4Da2 protein was degraded within 5 min. Several faint bands of <60 kDa corresponding to fragments of Vpb4Da2 were present during the first hour of digestion, but were no longer observed after 2 h. Vpb4Da2 in the control mixture lacking pancreatin remained intact after 24 h of incubation, indicating that the degradation of Vpb4Da2 in the reaction mixes is indeed due to proteolytic digestion of Vpb4Da2 by pancreatin.

Taken together, these results show that full length Vpb4Da2 is rapidly degraded by both pepsin and pancreatin.

Structural stability and bioactivity of Vpb4Da2 after exposure to heat

To assess stability after heating, a control sample was kept on wet ice (~0°C). Aliquots of the control and heated protein samples were subjected to SDS-PAGE and stained with Brilliant Blue G-Colloidal stain, with the extent of protein degradation assessed qualitatively. No visible degradation or decrease in band intensity was observed for Vpb4Da2 in the control, 25, 37, or 55°C treated samples at either 15 or 30 min. Some degradation and aggregation products were evident at 55°C at both 15 and 30 min. At 75°C, a decrease in the intensity of the main Vpb4Da2 band was evident, as well as degradation and aggregation products. The samples treated at 95°C for 15 and 30 min showed a significant loss of Vpb4Da2 band intensity and presence of degradation products, less than 10% remaining after the 30 min heat treatment. These data indicate that Vpb4Da2 is heat labile at temperatures greater than 75°C.

To assess the functional activity of Vpb4Da2 after heating, serial dilutions of the heated

protein samples were tested in a bioassay using WCR larvae similar to that described in Section 4.1.1.1. Control insects were fed a diet containing unheated Vpb4Da2 which had been maintained at ~0°C. The bioactivity for each sample was measured as a 7-day EC_{50} value, which is the Vpb4Da2 concentration that results in a 50% growth inhibition relative to control insects. At heating temperatures of 25 or 37°C for either 15 or 30 min, the activity of Vpb4Da2 remained similar to that of the unheated Vpb4Da2 (Table 7). At 55, 75 or 95°C, no dose-response relationship to the samples was observed, indicating that Vpb4Da2 activity was reduced to the extent that an EC_{50} value could not be established (<50% growth inhibition at the highest protein concentration tested). These results indicate that heating to temperatures of 55°C or above effectively destroys the functional activity of Vpb4Da2.

Table 7: Bioactivity of heat-treated Vpb4Da2 in a diet fed to insect larvae

Temperature	15 min heat treatment		30 min heat treatment	
	EC_{50} (µg Vpb4Da2/ml diet)	95% CI[b] (µg Vpb4Da2/ml diet)	EC_{50} (µg Vpb4Da2/ml diet)	95% CI[b] (µg Vpb4Da2/ml diet)
0°C (control)	10	7.4 – 14	10	7.4 – 14
25°C	14	11 – 18	10	6.1 – 16
37°C	14	8.0 – 26	12	6.4 – 24
55°C	N/A[a]	N/A	N/A[a]	N/A
75°C	N/A[a]	N/A	N/A[a]	N/A
95°C	N/A[a]	N/A	N/A[a]	N/A

a. The EC_{50} could not be calculated as <50% growth inhibition was observed at the highest protein concentration tested.
b. Confidence limits

4.1.3 Conclusion

A range of characterisation studies were performed on the plant-derived Mpp75Aa1.1 and Vpb4Da to confirm their identity, structure, biochemistry and function, as well their equivalence to the corresponding proteins produced in *E. coli*. For both Mpp75Aa1.1 and Vpb4Da, expression in MON95275 was highest in leaf tissue and lowest in pollen. Bioinformatic analyses showed Mpp75Aa1.1 and Vpb4Da2 did not share any meaningful homology with any known allergens or toxins. Both proteins were heat labile and susceptible to pepsin and pancreatin digestion. Taken together this indicates that the Mpp75Aa1.1 and Vpb4Da2 proteins are unlikely to be toxic or allergenic to humans.

4.2 Other Novel Substances

4.2.1   DvSnf7 dsRNA

The DvSnf7.1 cassette in MON95275 produces a 1014 nucleotide long RNA transcript containing two inverted repeat sequences that form a 240 base pair dsRNA (DvSnf7). This product, when ingested by corn rootworm larvae, is processed by the insect's RNAi machinery into 21-24-mer small interfering RNAs (siRNAs). These siRNAs bind to homologous mRNA transcripts in the insect midgut and downregulate the *DvSnf* gene, leading to insect death (Bolognesi et al. 2012).

The safety of the DvSnf7 dsRNA has been previously assessed in Application A1097 (FSANZ 2015). The available data do not indicate this dsRNA possesses different characteristics, or is likely to pose a greater risk, than other RNAi mediators naturally present in corn. A history of safe human consumption of RNAi mediators exists, including those with homology to human genes. The evidence published to date also does not indicate that dietary uptake of such RNA from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically relevant effect (FSANZ 2013).

5 Compositional analysis

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

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

5.1 Key components

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

5.2 Study design

MON95275 (F5F1 generation) and a non-GM control of similar genetic background (LH244 x HCL617) were grown and harvested from five field trial sites in the US during the 2019 growing season[15]. The sites were representative of corn growing regions suitable for commercial production. The field sites were established in a randomised complete block design with four replicates per site. Plants were grown under agronomic field conditions typical for each of growing region.

At maturity, grain was harvested from all plots and shipped to an analytical laboratory at ambient temperature, before being frozen at -20°C until analysis. Compositional analyses were performed on ground grain samples by Eurofins Food Integrity & Innovation. These analyses were based on internationally recognised procedures including official methods specified by the Association of Official Analytical Chemists (AOAC), the Analytical Oil Chemists' Society (AOCS), methods specified by the manufacturer of the equipment used for

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[15] The location of the five field trial sites: two sites in Illinois, and one site in each of Iowa, Indiana, and Missouri.

analysis, or other published scientific methods.

68 different analytes were measured in grain (see Figure 6 for a complete list). Moisture was also measured and used to convert the analyte values from fresh to dry weight. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, North Carolina). Analytes were expressed as either percent dry weight (% dw), mg/kg dw or as a percentage of total fatty acids (% total FA), as shown in Figure 7. A linear mixed model analysis of variance was applied on data combined across the five replicated field trial sites. For each analyte, 'descriptive statistics' (mean, standard error (SE), and range) were generated. Of the components measured, 15 had more than 50% of observations below the LOQ and were excluded from the statistical analyses (listed in grey in Figure 6), leaving a total of 53 components that were fully analysed in grain.

In assessing the statistical significance of any difference between MON95275 and the conventional control, a *p*-value of 0.05 was used. Any statistically significant differences were evaluated further. The magnitude of difference in mean values between MON95275 and the control were determined, and this difference was compared to the variation observed within the control grown at multiple sites. This variation was calculated from the range value of the control, and shows the natural variability of analytes in a plant with a similar background to MON95275 grown under the same agronomic and environmental conditions. In addition, the natural variation of analytes from publically available data was also considered (Egesel et al. 2003; Harrigan et al. 2009; Ridley et al. 2011; AFSI 2023). This takes into account variability present in non-GM corn cultivars due to a wider range of agronomic and environment conditions, as well as different genetic backgrounds. These data ranges assist with determining whether any statistically significant differences were likely to be biologically meaningful.

Key analyte levels (proximates and minerals) were also analysed in forage but the results are not included in this report. It is noted however that, in the combined site analysis, none of the analyte levels in MON95275 differed significantly from those of the control.

Figure 6. *Analytes measured in grain samples. Analytes listed in grey text were below the LOQ and excluded from statistical analysis. The 53 analytes listed in black text were analysed fully.*

5.3 Analyses of key components in grain

Of the 68 analytes measured in grain, mean values were provided for 53 analytes (Figure 6), and of these, there were 7 for which there was a statistically significant difference between corn line MON95275 and the control: palmitic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, calcium, and vitamin B_6. A summary of these 7 analytes is provided in Figure 7. For the complete data set, including values for the 46 analytes for which no statistically significant differences were found, refer to the Application dossier (pages 166 – 177).

For all 7 analytes where a statistically significant difference was found, the deviation of the MON95275 mean from the control mean was less than 7% (Figure 7a). However, as can be observed in Figure 7 (panels b-h), the MON95275 mean for each of these components was within the control range value, indicating that MON95275 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. In addition, the observed MON95275 means fall well within the natural variability seen in the publicly available data (grey bar, Figure 7, b-h). The differences reported here are therefore consistent with the normal biological variability that exists in corn.

Overall, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in MON95275 when compared with conventional non-GM corn cultivars already available in agricultural markets. Grain from MON95275 can therefore be regarded as equivalent in composition to grain from conventional non-GM corn.
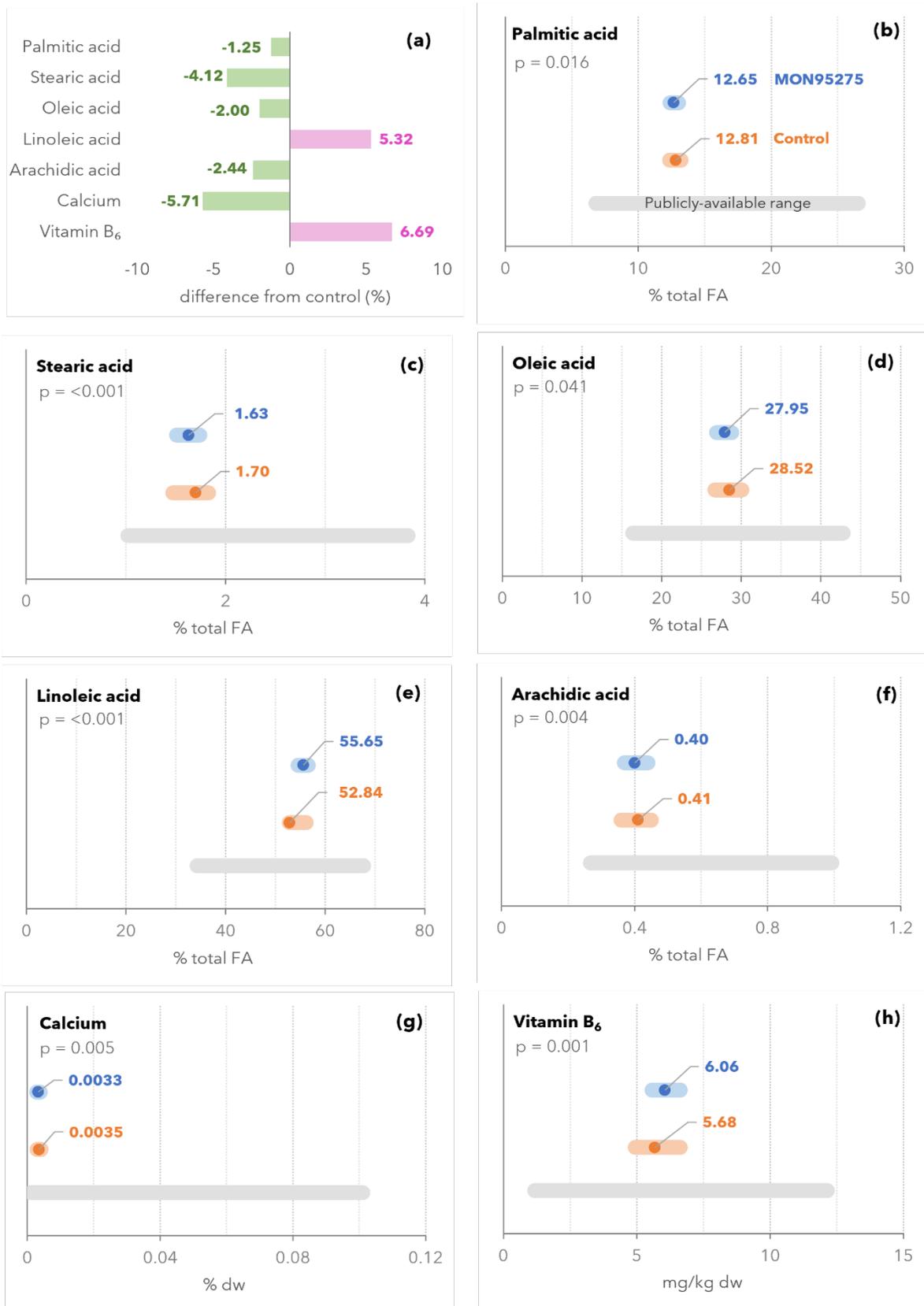
Figure 7. *Visual summary of statistically significant compositional differences between MON95275 and the conventional control.* **(a)** *Percentage deviation of the mean MON95275 value from the mean control value for each of the 7 analytes for which a statistically significant difference was found.* **(b) – (h)** *Measured means (dots) and ranges (coloured bars) for MON95275 (blue) and the conventional control (orange) for the 7 analytes as labelled. The grey bars represent the publicly-available range of values for each analyte. Note that the x-axes vary in scale and unit for each component.*

6   Nutritional impact

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

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

MON95275 is the result of genetic modifications to provide protection against coleopteran pests, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modification has not altered the nutrient composition of MON95275 compared with conventional non-GM corn cultivars. The introduction of food derived from MON95275 into the food supply is therefore expected to have negligible nutritional impact.

7   References

ACVM (2022) *Bacillus thuringiensis* products identified by searching the ACVM register for "thuringiensis" and recording those products that have been approved and registered. https://eatsafe.nzfsa.govt.nz/web/public/acvm-register, accessed December 2021

AFSI (2023) Crop Composition Database, Version 9.1, Agriculture & Food Systems Institute https://www.cropcomposition.org/, accessed January 2023

Anderson JE, Michno JM, Kono TJ, et al. (2016) Genomic variation and DNA repair associated with soybean transgenesis: a comparison to cultivars and mutagenized plants. BMC Biotechnol 16(1):41

APVMA (2022) *Bacillus thuringiensis* products identified by searching the APVMA website for "thuringiensis" and recording those products that have been approved and registered. https://portal.apvma.gov.au/pubcris, accessed December 2022

Babst, M, Katzmann, DJ, Estepa-Sabal, EJ, Meerloo, T, Emr, SD (2002) ESCRT-III: an endosome-associated heterooligomeric protein complex required for MVB sorting. Dev Cell 3(2):271-282

Barker RF, Idler KB, Thompson DV, Kemp JD (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol Biol 2(6):335-350

Barry GF, Kishore GM, Padgette SR, Stallings WC (2001) Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases. United States Patent 6,248,876

Bartholomaeus A, Parrott W, Bondy G, Walker K (2013) The use of whole food animal studies in the safety assessment of genetically modified crops: limitations and recommendations. Crit Rev Toxicol 43(Suppl 2):1-24

Baum, JA, Bogaert, T, Clinton, W, et al., (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25(11):1322-1326

Biggel M, Jessberger N, Kovac J, Johler S (2022) Recent paradigm shifts in the perception of the role of *Bacillus thuringiensis* in foodborne disease. Food Microbiol 105:104025

Bolognesi R, Ramaseshadri P, Anderson J, et al. (2012) Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). PLoS One 7(10):e47534

Bonis M, Felten A, Pairaud S, et al. (2021) Comparative phenotypic, genotypic and genomic analyses of *Bacillus thuringiensis* associated with foodborne outbreaks in France. PLoS One 16(2):e0246885

Bowen D, Yin Y, Flasinski S, et al. (2021) Cry75Aa (Mpp75Aa) Insecticidal proteins for controlling the western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), isolated from the insect-pathogenic bacterium *Brevibacillus laterosporus*. Appl Environ Microbiol 87(5):e02507-20

Bradshaw RA, Brickey WW, Walker KW (1998) N-terminal processing: the methionine aminopeptidase and N alpha-acetyl transferase families. Trends Biochem Sci 23(7):263-267

Brown, SM, Santino, CG (1997) Enhanced expression in plants. United States Patent 5,593,874

Casini, A, Christodoulou, G, Freemont, PS, Baldwin, GS, Ellis, T, MacDonald, JT (2014) R2oDNA designer: computational design of biologically neutral DNA sequences. ACS Synth Biol 3(8):525-528

CERA (2011) A review of the environmental safety of the Cry1Ab protein. Environ Biosafety Res 10(3):51-71

Coates BS, Walden KKO, Lata D, et al. (2023) A draft *Diabrotica virgifera virgifera* genome: insights into control and host plant adaption by a major maize pest insect. BMC Genomics 24(1):19

Crickmore N, Berry C, Panneerselvam S, Mishra R, Connor TR, Bonning BC (2021) A structure-based nomenclature for *Bacillus thuringiensis* and other bacteria-derived pesticidal proteins. J Invertebr Pathol 186:107438

Delaney B, Astwood JD, Cunny H, et al. (2008) Evaluation of protein safety in the context of agricultural biotechnology. Food Chem Toxicol 46(Suppl 2):71-97

Depicker, A, Stachel, S, Dhaese, P, Zambryski, P (1982) Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet 1(6):561-573

Egesel CÖ, Wong JC, Lambert R, Rocheford T (2003) Gene dosage effects on carotenoid concentration in maize grain. Maydica 48:183-190

Ehling-Schulz M, Lereclus D, Koehler TM (2019) The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. Microbiol Spectr 7(3)

FAOSTAT (2021) Online database of the Food and Agriculture Organisation of the United Nations. In. https://www.fao.org/faostat/en/#data, accessed January 2023

FSANZ (2013) Response to Heinemann *et al* on the regulation of GM crops and foods developed using gene silencing. Food Standards Australia New Zealand, Canberra.

FSANZ (2015) Application A1097 – Food derived from herbicide-tolerant and insect-protected corn line MON87411. Report prepared by Food Standards Australia New Zealand. https://www.foodstandards.gov.au/code/applications/Pages/A1097GMCornLineMON87411.aspx, accessed December 2022

Gilbertson L (2003) Cre-*lox* recombination: Cre-ative tools for plant biotechnology. Trends Biotechnol 21(12):550-555

Gray ME, Sappington TW, Miller NJ, Moeser J, Bohn MO (2009) Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annu Rev Entomol 54:303-321

GRDC (2017) Maize Northern Region - GrowNotes. In: Grains Research and Development Corporation. https://grdc.com.au/GN-Maize-North, accessed December 2022

Harrigan GG, Ridley WP, Miller KD, et al. (2009) The forage and grain of MON 87460, a drought-tolerant corn hybrid, are compositionally equivalent to that of conventional corn. J Agric Food Chem 57(20):9754-9763

Hernandez-Garcia CM, Finer, JJ (2014) Identification and validation of promoters and *cis*-acting regulatory elements. Plant Sci 217-218:109-119

Herrmann KM (1995) The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell 7(7):907-919

Hinnebusch AG (2011) Molecular mechanism of scanning and start codon selection in eukaryotes. Microbiol Mol Biol Rev 75(3):434-467

Hong HA, Duc le H, Cutting SM (2005) The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 29(4):813-835

Huang YF, Jordan WR, Wing RA, Morgan PW (1998) Gene expression induced by physical impedance in maize roots. Plant Mol Biol 37(6):921-930

Hunt AG (1994) Messenger RNA 3' end formation in plants. Curr Top Microbiol Immunol 326:151-177

Iurlina MO, Fritz R (2005) Characterization of microorganisms in Argentinean honeys from different sources. Int J Food Microbiol 105(3):297-304

Jeon JS, Lee S, Jung KH, Jun SH, Kim C, An G (2000) Tissue-preferential expression of a rice alpha-tubulin gene, *OsTubA1*, mediated by the first intron. Plant Physiol 123(3):1005-1014

Klee HJ, Muskopf YM, Gasser CS (1987) Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. Mol Gen Genet 210(3):437-442

Kouadio JL, Duff S, Aikins M, et al. (2021a) Structural and functional characterization of Mpp75Aa1.1, a putative beta-pore forming protein from *Brevibacillus laterosporus* active against the western corn rootworm. PLoS One 16(10):e0258052

Kouadio JL, Zheng M, Aikins M, et al. (2021b) Structural and functional insights into the first *Bacillus thuringiensis* vegetative insecticidal protein of the Vpb4 fold, active against western corn rootworm. PLoS One 16(12):e0260532

Kozak M (1989) Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. Mol Cell Biol 9(11):5134-5142

Kuluev BR, Chemeris AV (2007) Amplification and cloning of dahlia mosaic virus and carnation etched ring virus promoters. Russ J Genet 43(12):1413-1414

Labno A, Tomecki R, Dziembowski A (2016) Cytoplasmic RNA decay pathways - Enzymes and mechanisms. Biochim Biophys Acta 1863(12):3125-3147

Moar WJ, Evans AJ, Kessenich CR, et al. (2017) The sequence, structural, and functional diversity within a protein family and implications for specificity and safety: The case for ETX_MTX2 insecticidal proteins. J Invertebr Pathol 142:50-59

Nester EW, Thomashow LS, Metz M, Gordon M (2002) 100 years of *Bacillus thuringiensis*: a critical scientific assessment. American Academy of Microbiology, Washington (DC)

NZ EPA (2022) Approval: Lateral (*Brevibacillus laterosporus* strain 1951). Environmental Protection Authority, Wellington, New Zealand

Odell, JT, Nagy, F, Chua, NH (1985) Identification of the DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313(6005):810-812

OECD (2002) Consensus document on compositional considerations for new varieties of maize (*Zea mays*): Key food and feed nutrients, anti-nutrients, toxicants and secondary plant metabolites. ENV/JM/MONO(2002)25. Organisation for Economic Cooperation and Development, Paris

OECD (2003) Considerations for the Safety Assessment of Animal Feedstuffs Derived from Genetically Modified Plants. Series on the Safety of Novel Foods and Feeds, No. 9. Organisation for Economic Cooperation and Development, Paris

OGTR (2008) The Biology of *Zea mays* L. ssp *mays* (maize or corn). Office of the Gene Technology Regulator, Canberra, Australia

Palma L, Munoz D, Berry C, Murillo J, Caballero P (2014) *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins (Basel) 6(12):3296-325

Panda AK, Bisht SS, DeMondal S, Senthil Kumar N, Gurusubramanian G, Panigrahi AK (2014) *Brevibacillus* as a biological tool: a short review. Antonie Van Leeuwenhoek 105(4):623-639

Ranum P, Pena-Rosas JP, Garcia-Casal MN (2014) Global maize production, utilization, and consumption. Ann NY Acad Sci 1312:105-112

Raymond B, Federici BA (2017) In defense of *Bacillus thuringiensis*, the safest and most successful microbial insecticide available to humanity – a response to EFSA. FEMS Microbiol Ecol 93(7):e_fix084

Ridley WP, Harrigan GG, Breeze ML, Nemeth MA, Sidhu RS, Glenn KC (2011) Evaluation of compositional equivalence for multitrait biotechnology crops. J Agric Food Chem 59(11):5865-5876

Rigden DJ, Mello LV, Galperin MY (2004) The PA14 domain, a conserved all-beta domain in bacterial toxins, enzymes, adhesins and signaling molecules. Trends Biochem Sci 29(7):335-339

Rochester, DE, Winer, JA, Shah, DM (1986) The structure and expression of maize genes encoding the major heat shock protein, hsp70. EMBO J 5(3):451-458

Rose AB (2008) Intron-mediated regulation of gene expression. Curr Top Microbiol Immunol 326:277-290

Ruiu L (2013) *Brevibacillus laterosporus*, a pathogen of invertebrates and a broad-spectrum antimicrobial species. Insects 4(3):476-92

Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. Mol Gen Genet 234(1):49-59

Salomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO J 17(20):6086-6095

Sarkar PK, Hasenack B, Nout MJ (2002) Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala). Int J Food Microbiol 77(3):175-186

Schnepf E, Crickmore N, Van Rie J, et al. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62(3):775-806

Shida O, Takagi H, Kadowaki K, Komagata K (1996) Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int J Syst Bacteriol 46(4):939-46

Siderov V, Duncan D (2009) *Agrobacterium*-mediated maize transformation: immature embryos versus calluses. Pages 47-58 in Methods in Molecular Biology: Transgenic Maize - Methods and Protocols. M.P. Scott (ed.). Humana Press, Inc, Totowa, New Jersey

Strickberger MW (1976) Probability and statistical testing in genetics, 2nd ed. MacMillan Publishing Company, New York

Thomas K, Aalbers M, Bannon GA, et al. (2004) A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. Regul Toxicol Pharmacol 39(2):87-98

USDA (2022) Grain: World Markets and Trade. Available online at https://www.fas.usda.gov/psdonline/circulars/grain.pdf, Accessed December 2022

Yang L, Wang C, Holst-Jensen A, Morisset D, Lin Y, Zhang D (2013) Characterization of GM events by insert knowledge adapted re-sequencing approaches. Sci Rep 3:2839

Yin Y, Flasinski S, Moar W, et al. (2020) A new *Bacillus thuringiensis* protein for Western corn rootworm control. PLoS One 15(11):e0242791

Zastrow-Hayes GM, Lin H, Sigmund AL, et al. (2015) Southern-by-Sequencing: a robust screening approach for molecular characterization of genetically modified crops. Plant Genome 8(1): e2014.08.0037

Zambryski, P, Depicker, A, Kruger, k, Goodman, HM (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. J Mol Appl Genet 1(4):361-370

Zhang W, Subbarao S, Addae P, et al. (2003) Cre/*lox*-mediated marker gene excision in transgenic maize (*Zea mays* L.) plants. Theor Appl Genet 107(7):1157-1168

Appendix 1

Flowchart showing the development process used for creation of the MON95275 corn line

```
┌─────────────────────────────────────────────────────────────────┐
│ Assembled Agrobacterium binary plasmid vector PV-ZMIR525664 and   │
│ transferred to Agrobacterium tumefaciens strain ABI               │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│ Transformed LH244 (a maize line for more efficient transformation)│
│ immature embryos with PV-ZMIR525664 in Agrobacterium tumefaciens  │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│ Selected transformants (R0 plants) containing the selectable      │
│ marker (cp4 epsps expression cassette) and generated rooted       │
│ shoots from the transformed callus tissues                        │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│ Evaluated R1 plants by PCR and selected the transformed plants    │
│ for the homozygous presence of the T-DNA                          │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│ Selected R2 plants were crossed with the Cre recombinase          │
│ expressing line                                                   │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│ Identified and selected plants lacking the cp4 epsps and cre      │
│ genes in F2 plants respectively                                   │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│ Evaluated plants (subsequent generations) for insert integrity    │
│ (via molecular analyses), insect control efficacy and other       │
│ phenotypic characteristics                                        │
└─────────────────────────────────────────────────────────────────┘
                                │
                                ▼
┌─────────────────────────────────────────────────────────────────┐
│ Selected MON 95275 as lead event and further evaluated its        │
│ progeny in laboratory and field assessments for T-DNA insert      │
│ integrity, trait efficacy, agronomic/phenotypic characteristics,  │
│ compositional equivalence, and absence of all other vector DNA    │
│ and PV-ZMOO513642 DNA                                             │
└─────────────────────────────────────────────────────────────────┘
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Appendix 2

PV-ZMIR525664-derived genetic elements in T-DNA region

Genetic element	Relative position	Size (bp)	Source	Description, Function & Reference	Present in MON95275
B[1]- Right border region	1-331	331	*Agrobacterium tumefaciens*	Sequence used to transfer the T-DNA region into the host genome (Depicker et al. 1982; Zambryski et al. 1982)	Yes[rl]
Intervening sequence	332-436	105	Synthetic		Yes
***DvSnf7.1* expression cassette**					
T[2]-*E9*	437-1069	633	*Pisum sativum*	3' untranslated region of the *rbcS* gene family that directs polyadenylation of the DvSNf7 mRNA	Yes
Intervening sequence	1070-1098	29	Synthetic		Yes
DvSnf7[3]	1099-1338	240	*Diabrotica virgifera virgifera*	Partial coding sequence of the *Snf7* gene: encodes the Snf7 subunit of the ESCRT-III complex (Babst et al. 2002; Baum et al. 2007)	Yes
Intervening sequence	1339-1488	150	Synthetic		Yes
DvSnf7[3]	1489-1728	240	*Diabrotica virgifera virgifera*	Partial coding sequence of the *Snf7* gene: encodes the Snf7 subunit of the ESCRT-III complex (Babst et al. 2002; Baum et al. 2007)	Yes
Intervening sequence	1729-1764	36	Synthetic		Yes
I[4]-*Hsp70*	1765-2568	804	*Zea mays*	Intron and flanking exon sequence of the *hsp70* gene, involved in regulating gene expression (Rochester et al. 1986; Brown and Santino 1997)	Yes
Intervening sequence	2569-2574	6	Synthetic		Yes
P[5]-*35S*	2575-3150	576	Cauliflower mosaic virus (CaMV)	Promoter and leader of the 35S RNA that directs transcription (Odell et al. 1985)	Yes
E[6]-*pIIG-Zm1*	3151-4066	916	*Zea mays*	Optimised enhancer sequence of the *pIIG* gene. Directs transcription in plant cells. (Huang et al. 1998)	Yes
Intervening sequence	4067-4072	6	Synthetic		Yes
S[7]-*Isr-1*	4073-5291	1219	Synthetic	Non-coding intergenic sequence that minimises effects on gene expression from neighbouring cassettes (Casini et al. 2014)	Yes
Intervening sequence	5292-5312	21	Synthetic		Yes
***mpp75Aa1.1* expression cassette**					
E-*DaMv-1*	5313-5634	322	Dalia mosaic virus (DaMV)	Enhancer sequence from promoter region that enhances transcription in plant cells (Kuluev and Chemeris 2007)	Yes
Intervening sequence	5635-5647	13	Synthetic		Yes
P-*RCc3-Td1*	5648-6478	831	*Tripsacum dactyloides*	Promoter and leader of the *Rcc3* gene that directs transcription in plant cells (Hernandez-Garcia and Finer 2014)	Yes

Genetic element	Relative position	Size (bp)	Source	Description, Function & Reference	Present in MON95275
I-14-3-3c-Si1	6479-6583	105	*Setaria italica*	Intron from a putative *14-3-3c* gene that regulates gene expression (Rose 2008)	Yes
Intervening sequence	6584-6604	21	Synthetic		Yes
CS[8]-Mpp75Aa1.1	6605-7492	888	*Brevibacillus laterosporus*	Codon-optimized coding sequence for the Mpp75Aa1.1 protein that provides insect resistance (Bowen et al. 2020)	Yes
Intervening sequence	7493-7509	17	Synthetic		Yes
T-HSP-Cl1	7510-8070	561	*Coix lacryma-jobi*	3'UTR sequence of a *Hsp* gene that directs polyadenylation of the *mpp75Aa1.1* mRNA (Hunt 1994)	Yes
Intervening sequence	8071-8096	26	Synthetic		Yes
vpb4Da2 expression cassette					
T-SAM1-Si1	8097-8531	435	*Setaria italica*	3'UTR sequence of an S-adenosylmethionine synthetase 1 gene that directs polyadenylation of the *vpb4Da2* mRNA (Hunt 1994)	Yes
Intervening sequence	8532-8538	7	Synthetic		Yes
CS-Vpb4Da2	8539-11352	2814	*Bacillus thuringiensis*	Codon optimised coding sequence for the Vpb4Da2 protein that provides insect resistance (Yin et al 2020)	Yes
Intervening sequence	11353-11378	26	Synthetic		Yes
I-Act-Si1	11379-12743	1365	*Setaria italica*	Intron from an actin gene involved in regulating gene expression (Rose 2008)	Yes
Intervening sequence	12744-12751	8	Synthetic		Yes
P-Ltp-Zm1	12752-14045	1294	*Zea mays*	Promoter and leader of a lipid transfer protein gene that directs transcription in plant cells (Hernandez-Garcia and Finer 2014)	Yes
E-DaMV-2	14046-14541	496	Dalia mosaic virus (DaMV)	Enhancer from a promoter region that enhances transcription in plant cells (Kuluev and Chemeris 2007)	Yes
Intervening sequence	14542-14685	144	Synthetic		Yes
loxP	14686-14719	34	Bacteriophage P1	Cre recombinase recognition site (Russell et al. 1992)	Yes
Intervening sequence	14720-14725	6	Synthetic		**No***
cp4 epsps expression cassette					
P-TubA	14726-16906	2181	*Oryza sativa*	Promoter, 5' UTR and intron sequences of the *OsTubA* gene family encoding α-tubulin that directs transcription in plant cells (Jeon et al. 2000)	**No**
Intervening sequence	16907-16910	4	Synthetic		**No**
TS[9]-CTP2	16911-17138	228	*Arabidopsis thaliana*	Coding sequence of a transit peptide from the *ShkG* gene that directs the EPSPS protein to chloroplasts (Klee et al. 1987; Herrmann 1995)	**No**

Genetic element	Relative position	Size (bp)	Source	Description, Function & Reference	Present in MON95275
CS-*cp4 epsps*	17139-18506	1367	*Agrobacterium* sp. strain CP4	Coding sequence of the *aroA* gene for the CP4 EPSPS protein that provides tolerance to glyphosate (Barry et al. 2001)	**No**
Intervening sequence	18506-18513	8	Synthetic		**No**
T-*TubA*	18514-19095	582	*Oryza sativa*	3'UTR sequence of the *OsTubA* gene that directs polyadenylation of mRNA (Jeon et al., 2000)	**No**
Intervening sequence	19096-19101	6	Synthetic		**No**
*lox*P	19102-19135	34	Bacteriophage P1	Cre recombinase recognition site (Russell et al. 1992)	**No**
Intervening sequence	19136-19170	35	Synthetic		Yes
B-Left border region	19171-19612	442	*Agrobacterium tumefaciens*	Sequence used to transfer the T-DNA region into the host genome (Barker et al. 1983)	Yes[rl]

* Bases 14720 through 19135 of the PV-ZMIR525664 T-DNA was excised from an intermediary line in the development of MON95275. This removed DNA sequences in the selectable marker cassette (P-*TubA*, TS-*CTP2*, CS-*cp4 epsps*, and T-*TubA*) and one *lox*P site. One 34 bp *lox*P site remained in MON95275 (bases 14686--14719).

[1] Border, [2] Terminator Sequence, [3] Partial, [4] Intron, [5] Promoter, [6] Enhancer, [7] Spacer, [8] Coding Sequence, [9] Targeting Sequence

[rl] The sequences of both the left and right border regions in MON95275 were truncated compared to those in PV-ZMIR525664

Appendix 3

PV-ZMOO513642 plasmid map used to create the Cre recombinase-expressing line