FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

7 January 2025
324-25

Supporting document 1

Safety assessment – Application A1310

Food derived from insect-protected soybean line MON94637

Executive summary

Background

Application A1310 seeks approval for the sale and use of food derived from soybean line MON94637 that has been genetically modified (GM) for protection against lepidopteran insect pest.

Protection is conferred by the expression of *cry1A.2* and *cry1B.2* genes encoding two novel insecticidal proteins: Cry1A.2 and Cry1B.2, respectively. The Cry1A.2 and Cry1B.2 are chimeric proteins comprised of multiple domains from different Cry proteins from the soil bacterium *Bacillus thuringiensis*. This is the first time Food Standards Australia New Zealand (FSANZ) has assessed the Cry1A.2 and Cry1B.2 proteins.

This safety assessment addresses food safety and nutritional issues associated with 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

Soybean has a long history of safe use in the food supply. Soybean oil is widely used in cooking and as an ingredient in a wide range of manufactured products. Soybean grains are also used to make soy milk, soy sauce, soy lecithin and meat substitutes such as tofu and tempeh.

Molecular characterisation

The genes encoding Cry1A.2 (*cry1A.2*) and Cry1B.2 (*cry1B.2*) were introduced into soybean line MON94637 via *Agrobacterium*-mediated transformation. Detailed molecular analyses indicate a single copy of each of the two gene cassettes is present at a single insertion site in the MON94637 genome. There are no extraneous plasmid sequences or antibiotic resistance genes present in this line.

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

Characterisation and safety assessment of new substances

Both Cry1A.2 and Cry1B.2 proteins are expressed throughout the plant (except Cry1B.2 which was not detected in root tissue). Expression levels are low in grain.

A range of characterisation studies confirmed the identity of the plant-expressed Cry1A.2 and Cry1B.2 proteins and their equivalence with the corresponding proteins produced in a bacterial expression system. The plant-derived and bacterially-derived proteins had the expected molecular weight, amino acid sequence, immunoreactivity, lack of glycosylation and enzyme activity.

Both Cry1A.2 and Cry1B.2 proteins are rapidly degraded and heat inactivated, based on studies submitted with this application. Bioinformatics studies on the two proteins confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens. Taken together, the evidence supports the conclusion that Cry1A.2 and Cry1B.2 are not toxic or allergenic to humans.

Compositional analyses

Detailed compositional analyses were performed on MON94637. Statistically significant differences in mean values were found between grain from MON94637 and the control for 3 of the 47 analytes evaluated, however these differences were within the range established for existing commercial non-GM soybean varieties. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in grain from MON94637 compared to non-GM soybean varieties available on the market.

Conclusion

No potential public health and safety concerns have been identified in the assessment of insect-protected soybean line MON94637. On the basis of the data provided in the present application and other available information, food derived from MON94637 is considered to be as safe for human consumption as food derived from non-GM soybean varieties.

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List of Abbreviations

Abbreviation	Definition
AFSI	Agriculture and Food Systems Institute
BLAST	Basic Local Alignment Search Tool
BLOSUM	BLOcks SUbstitution Matrix
bp	Base pair
CCI	Confidential Commercial Information
COMPARE	COMprehensive Protein Allergen Resource
DNA	Deoxyribonucleic acid
DW/dw	Dry weight
ECL	Enhanced chemiluminescence
ELISA	Enzyme Linked Immunosorbent Assay
FA	Fatty acid
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia New Zealand
GM	Genetically modified
h	Hours
kDa	Kilodalton
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
Min	Minutes
MT	Million tonnes
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NGS	Next Generation Sequencing
OECD	Organisation for Economic Cooperation and Development
OGTR	Office of the Gene Technology Regulator
ORF	Open reading frame

Abbreviation	Definition
OSL	Over-season leaf
PCR	Polymerase chain reaction
RB	Right border
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SBL	Soybean looper
T-DNA	Transfer DNA
µg	Microgram(s)
US	Unites States
USDA	United States Department of Agriculture
UTR	Untranslated region

1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from Bayer CropScience Pty Ltd to vary Schedule 26 in the Australia New Zealand Food Standards Code. The variation is to include food derived from a new genetically modified (GM) soybean line MON94637, with the OECD Unique Identifier MON-94637-8. This soybean line has been modified for protection against lepidopteran insect pests.

Protection is conferred by the expression of *cry1A.2* and *cry1B.2* genes encoding two novel insecticidal proteins: Cry1A.2 and Cry1B.2, respectively. The Cry1A.2 and Cry1B.2 are chimeric proteins comprised of multiple domains from different Cry proteins derived from the soil bacterium *Bacillus thuringiensis*. FSANZ has assessed numerous previous applications for crops containing Cry proteins derived from *B. thuringiensis*. This is the first time FSANZ has assessed the Cry1A.2 and Cry1B.2 proteins, specifically.

If approved, food derived from soybean line MON94637 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 soybean (*Glycine Max* (L.)), from the family Leguminosae. The non-GM soybean variety A3555 was used as the parental variety for the genetic modification described in this application and served as the conventional control for the purposes of comparative assessment with MON94637.

Soybean has a long history of safe human and animal consumption, having first been cultivated in northern China as early as 5000 years ago (Liu 2004). The commodity is the leading oilseed crop in the world, with total global production reaching 394.71 MT in 2023/2024 (USDA 2024). Brazil is the world's largest producer of soybean, producing 153 MT, followed by the United States (US), producing 113.27 MT in 2023/2024 (USDA 2024). Of the soybeans grown in Brazil and the US, the proportion of GM soybeans is estimated at 99% and 95% respectively (ASA 2024).

Soybean production in Australia is comparatively minor, totalling 0.065 MT in 2023/2024 (USDA 2024), while New Zealand has no commercial soybean production. Australia and New Zealand are net importers of soybeans, with 2788.88 tonnes and 2352.68 tonnes imported respectively in 2022 (FAOSTAT 2024). No GM soybean lines are currently grown commercially in Australia.[1]

Whole soybeans are used to produce soy milk, tofu, and soy sprouts, as well as fermented foods including miso, soy sauce, natto and tempeh. Soybeans may also be eaten with minimal processing, for example, as edamame, in which immature soybeans are boiled whole in their pods and served with salt. Soybean grains are processed into two major products: oil and meal. Soybean oil is the second most consumed vegetable oil worldwide and accounts for 29% of global vegetable oil consumption (American Soybean Association 2023). It is used in a variety of manufactured foods, including cooking oil, shortening, margarine, salad dressings, frozen desserts and confectionery products. Soybean meal is a good source of protein and is primarily processed into livestock feed (pet and poultry food) and protein products such as soy flour, concentrates and isolates.

[1] Information on approved commercial releases of GM crops in Australia can be found on the website of the Office of the Gene Technology Regulator: https://www.ogtr.gov.au/

Soybeans contain numerous bioactive phytochemicals, such as isoflavones and tocopherols (vitamin E) (Liu 2004), and are used as a source of these compounds for dietary supplements. Unprocessed (raw) soybean grain products are not suitable for food use, due to the presence of anti-nutrients, such as phytic acid and lectins (OECD 2012). The heat applied during processing inactivates these anti-nutrients.

2.2 Donor organisms

2.2.1 Bacillus thuringiensis

The genes encoding the Cry1A.2 and Cry1B.2 proteins are derived from *B. thuringiensis*, a facultative anaerobic, gram-positive, spore-forming bacterium found in soil (Palma et al. 2014). *B. thuringiensis* expresses a number of insecticidal proteins, including the well-characterised Cry proteins. While highly specific and toxic to their target insects, Cry proteins are innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo et al. 2007).

B. thuringiensis strains have a long history of use for the control of agricultural insect pests[2] (Nester et al. 2002; CERA 2011). Currently, there are approximately 44 biopesticide products based on *B. thuringiensis* registered in Australia (APVMA 2024) and 10 in New Zealand (ACVM 2024). The widespread use of *B. thuringiensis* in agriculture, along with its ubiquitous presence in soil and on vegetation, mean humans have a long history of safe exposure through food.

B. thuringiensis has been linked to human diarrheal illness due to its close relationship with the species *Bacillus cereus*. Some *B. cereus* strains contain enterotoxin genes and are pathogenic to humans (Ehling-Schulz et al. 2019; Biggel et al. 2022), However, it is unlikely *B. thuringiensis* subspecies or strains are causal agents of food-induced diarrhoea (Raymond and Federici 2017; Biggel et al. 2022).

2.2.2 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of MON94637 (refer to Table 1 and Appendix 2). These genetic elements are non-coding sequences that are used to regulate the expression of the inserted genes.

[2] Since 1938 in France and 1961 in the United States

3 Molecular characterisation

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

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

3.1 Transformation method

To create the MON94637 soybean line, the conventional soybean variety A3555 was transformed using the binary plasmid vector PV-GMIR527237 (Figure 1). This plasmid contains two separate transfer DNAs (T-DNAs):

- T-DNA I – contains the two trait expression cassettes; and
- T-DNA II – contains the selectable marker cassettes *splA* and *aadA* .

While both T-DNAs were inserted into the soybean genome during transformation, plants that contained only T-DNA I, and not T-DNA II, were isolated for further development. The transformation and development methodology is outlined in the flowchart in Appendix 1 and summarised below.

Transformation of A3555 was achieved by co-culturing meristem explants with *Agrobacterium tumefaciens* containing the PV-GMIR527237 plasmid. The meristem explants were then placed on selective media containing spectinomycin, carbenicillin, cefotaxime and timentin. Spectinomycin inhibits the growth of untransformed plant cells, while carbenicillin, cefotaxime and timentin suppress the growth of excess *Agrobacterium*.

Putative transformants (R0) with normal phenotypes were selected and screened for the presence of the T-DNA I insert unlinked to the T-DNA II insert, and for the absence of the vector backbone. Selected R0 plants were transferred to soil and self-pollinated to produce R1 seed, then evaluated phenotypically for the presence of *splA* (which produces a wrinkled seed phenotype) and by polymerase chain reaction (PCR) to identify the presence/absence of the *aadA* gene (confers resistance to spectinomycin) and were used to eliminate plants containing the T-DNA II insert. R1 plants that were homozygous for the T-DNA I insert, and lacked the T-DNA II insert, were selected for further development.

Following the evaluation of insert integrity, gene expression, phenotypic characteristics and agronomic performance, soybean line MON94637 was selected.

3.2 Detailed description of inserted DNA

Soybean line MON94637 contains the T-DNA I from the PV-GMIR527237 plasmid (Figure 1) and includes the *cry1A.2 and cry1B.2* expression cassettes.

Information on these two expression cassettes is summarised in Table 1. Additional detail, including the expression cassettes contained in the T-DNA II region from the PV-GMIR527237 plasmid (used as selectable markers during transformation), and intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in Appendix 2.

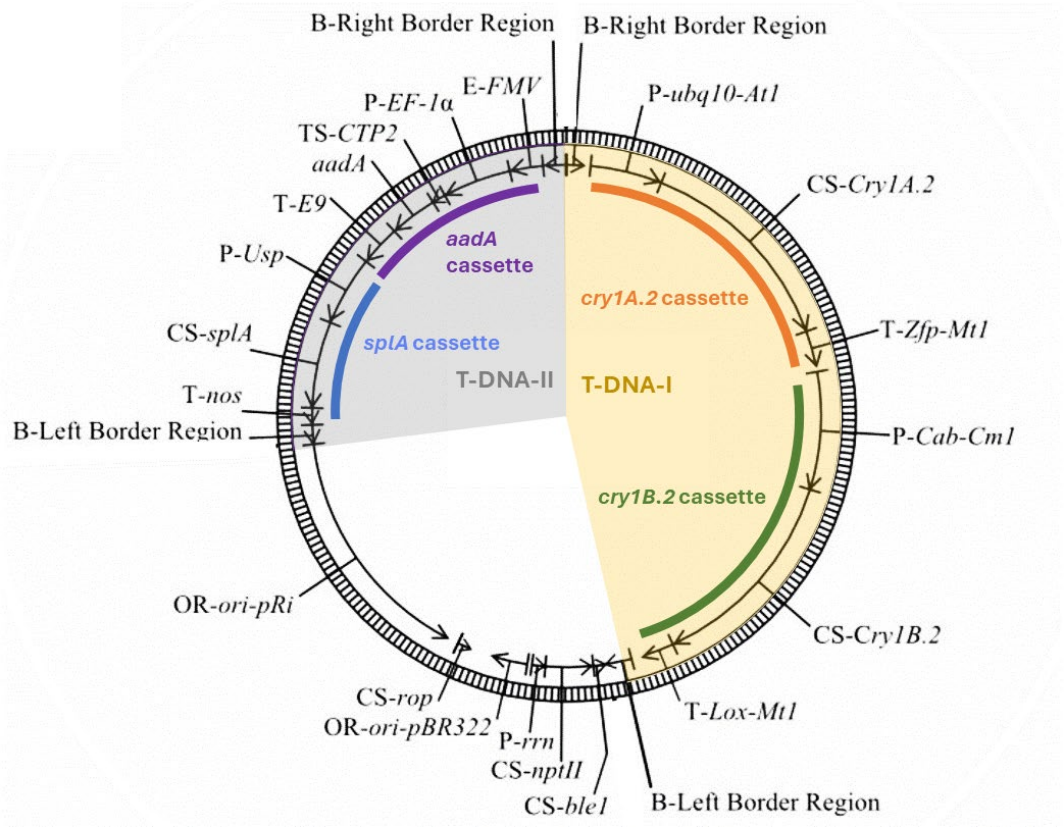

Figure 1: *Map of plasmid PV-GMIR527237 (26,558 bp). The T-DNA I region comprising the cry1A.2 and cry1B.2 expression cassettes is shaded yellow. The T-DNA II region comprising the splA and aadA selectable marker cassettes, which does not form part of the insert in MON94637, is shaded grey. The plasmid backbone is unshaded.*

Table 1: Expression cassettes contained in the MON94637 insert

	Promoter	Intron / 5'UTR region	Coding sequence	Terminator
***cry1A.2* expression cassette**	Promoter region from *Arabidopsis* thaliana (Arabidopsis) polyubiquitin gene (*Ubi10*)	Intron region from Arabidopsis *polyubiquitin gene*	Chimeric gene comprised of domains from *cry1Ah*, *cry1Ac*, *cry1Ca* from *B. thuringiensis*	Terminator region of a gene encoding a putative zinc finger protein (*Zfp*) from *Medicago truncatula* (barrel medic)
***cry1B.2* expression cassette**	Promoter region of a chlorophyll a/b-binding protein gene (*Cab*) from *Cucumis melo* (melon)	5'UTR region of a *Cab* protein from *c. melo*	Chimeric gene comprised of domains from *cry1Be*, *cry1Ka2*, *cry1Ab* from *B. thuringiensis*	Terminator region of lipoxygenase gene (*lox*) from *M. truncatula*

3.3 Development of the soybean line from the original transformant

A breeding program was undertaken for the purpose of:

- obtaining generations suitable for analysing the characteristics of soybean line MON94637; and
- ensuring that the MON94637 event is incorporated into elite lines for commercialisation.

A breeding history diagram depicting how MON94637 was derived from the original transformant was provided in the application as Confidential Commercial Information (CCI). While full details of the breeding history cannot be provided in this public report, FSANZ has given regard to this information in its assessment.

Table 2 indicates the specific generations and controls used in the characterisation of MON94637.

Table 2: MON94637 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Number of integration sites	Section 3.4.1	R3	A3555
Absence of backbone and other sequences	Section 3.4.2	R3	A3555
Insert integrity and site of integration	Section 3.4.3	R3	A3555
Genetic stability	Section 3.4.4.1	R3, R4, R5, R6, R7	A3555
Mendelian inheritance	Section 3.4.4.2	F2, F3, F4	N/A
Expression of phenotype over several generations	Section 3.4.4.2	R3, R4, R5, R6, R7	A3555
Compositional analysis	Section 5	R6	A3555

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

A range of analyses were undertaken to characterise the genetic modification in MON94637. 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.

Seed-derived genomic DNA (gDNA) from five generations of MON94637 (R3, R4, R5, R6 and R7) and from the conventional control (A3555) 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-GMIR527237 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 MON94637 and the control, with a depth of coverage ≥75x and an adequate level of sensitivity[3].

3.4.1 Number of integration site(s)

NGS reads from MON94637 (R3) that mapped to the intended T-DNA I insert were analysed and two unique insert-flank junction sites were identified. Each comprised the inserted T-DNA I border sequence joined to a flanking sequence in the soybean genome. This indicates that a single copy of the T-DNA I insert has been integrated into the genome of MON94637 (Figure 2). As expected, no junction sequences were detected in the A3555 control.

[3] 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 MON94637 and the controls was comprehensive.

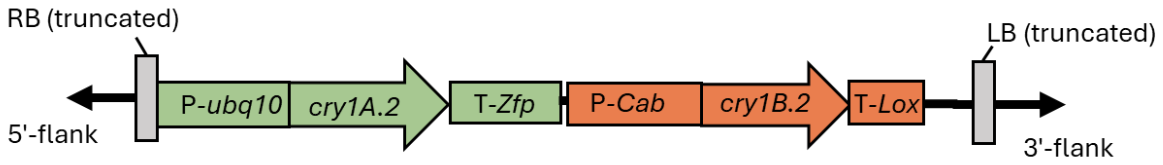

Figure 2*: T-DNA I insert (12,240 bp) present in MON94637.*

3.4.2 Absence of backbone and other sequences

Alignment of NGS reads from MON94637 (R3) and the PV-GMIR527237 transformation plasmid confirmed that the MON94637 genome does not contain any sequences from T-DNA II or the PV-GMIR527237 backbone, including antibiotic resistance genes.

When NGS reads from the A3555 control were aligned with the transformation plasmid sequence, a small number of reads mapped to parts of the T-DNA I and plasmid backbone. This low number of reads 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).

3.4.3 Insert integrity and site of integration

Locus-specific PCR and DNA sequence analysis of gDNA from MON94637 showed that a single copy of T-DNA I from PV-GMIR527237 was integrated into the host genome and the organisation of the genetic elements within the insert is as expected (Figure 2). Both border regions in the MON94637 insert had small terminal truncations relative to these regions in PV-GMIR527237. These changes would not 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, no T-DNA II elements were present. These results were fully consistent with the NGS dataset.

To examine the T-DNA I insertion site, PCR primers flanking the insertion site were used to amplify gDNA from the A3555 conventional control. Comparing the sequence of the PCR product to the sequence generated from the flanking regions of MON94637 identified a 14 base deletion of the soybean genomic DNA that occurred during T-DNA integration. All other flanking sequences in MON94637 were identical to those in the control. 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 *cry1A.2 or cry1B.2* genes.

3.4.4 Stability of the genetic changes in soybean line MON94637

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 MON94637 by evaluating seed-derived gDNA from five breeding generations of MON94637 (R3, R4, R5, R6 and R7). Control gDNA was isolated from the non-GM parental line A3555. The analysis showed that the two insert-flank junction sequences present in the R3 generation (section 3.4.1) were identical in each of the four additional generations. No other junction sequences were present. The

consistency of these results across the breeding generations tested demonstrates that the single T-DNA I insert is stably integrated in MON94637.

3.4.4.2 Phenotypic stability

Mendelian inheritance

Since the inserted DNA resides at a single locus within the MON94637 genome, the inserted DNA would be expected to be inherited according to Mendelian inheritance principles. To produce the generations for segregation analysis, homozygous positive R3 plants containing the T-DNA I insert were crossed with a proprietary elite line lacking the insert, producing hemizygous F1 seed. The F1 plants were self-pollinated to produce F2 seed. The inheritance of the T-DNA I insert was assessed by Qualitative End Point TaqMan PCR assay in the F2 generation, as well as in the subsequent F3 and F4 generations. A chi-square (χ^2) analysis was undertaken to confirm the segregation and stability of the insert.

According to Mendelian principles, the predicted segregation ratio in all generations was 1:2:1 (homozygous positive: hemizygous positive: homozygous negative). The results presented in Table 3 demonstrate the expected segregation ratio for each generation. This confirms that the inserted DNA is present at a single locus in the MON94637 genome and is inherited predictably according to Mendelian inheritance rules.

Table 3: Segregation of T-DNA I in three generations of MON94637

Generation	Expected segregation ratio	Observed number of plants (expected number)				Statistical analysis	
		Homozygous Positive	Hemizygous positive	Homozygous Negative	Total	χ^2	*P-value*
F2	1:2:1	87 (95)	192 (190)	101 (95)	380	1.07	0.585
F3	1:2:1	133 (140.5)	274 (281)	155 (140.5)	562	2.07	0.355
F4	1:2:1	107 (94.75)	188 (189.5)	84 (94.75)	379	2.82	0.245

Expressed phenotype over several generations

The expression of the Cry1A.2 and Cry1B.2 proteins in five generations of MON94637 (R3, R4, R5, R6 and R7) was examined. Western blot analysis was conducted on seed tissue from each generation, with seed tissue from the conventional line A3555 used as a negative control, and *B. thuringiensis*-produced versions of the proteins used as positive controls. In all five breeding generations, the Cry1A.2 and Cry1B.2 proteins migrated indistinguishably from the corresponding positive controls on the same Western blot. These data support the conclusion that the Cry1A.2 and Cry1B.2 proteins are stably expressed over multiple generations.

3.4.5 Open reading frame (ORF) analysis

A bioinformatic analysis of the MON94637 insert, as well as the flanking DNA regions, was undertaken to identify any novel reading frames (RFs) which had been created in MON94637 as a result of the insertion of T-DNA I, and whether any of these putative peptides have the potential for allergenicity or toxicity.

Sequences spanning the right and left insert-flank junctions of MON94637 were translated *in*

silico from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames.[4] Similarly, the entire MON94637 insert DNA was translated in all six reading frames. A total of 9 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 other than the intended Cry1A.2 and Cry1B.2 proteins 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 MON94637 insert sequence were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource (COMPARE[5]) database, from the Health and Environmental Science Institute. At the date (January 2023) of the search, there were 2,631 sequences in the allergen database (AD_2023). Sequences were also compared to the GenBank all protein database (PRT_2023), downloaded from the National Centre for Biotechnology Information (NCBI[6]), which contained 242,830,012 sequences at the date of download (January 2023).

Three types of analyses were performed for this comparison:

(a) A FASTA search algorithm (v36.3.5d) (Pearson and Lipman 1988), was used to identify alignments between the query sequences and the COMPARE database, using a BLOSUM50 scoring matrix, which identifies blocks of residues with at least 50% sequence identity (Henikoff and Henikoff 1992). Only matches with E-scores of $\leq 1\times10^{-5}$ 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 9 putative peptides present in the 5' and 3' insert-flank junctions with database sequences did not identify any matches. Similarly, no matches were identified when the translated 6 reading frames in the MON94637 insert DNA were aligned with the allergen (AD_2023) and all protein database (PRT_2023).

The results of this analysis support the conclusion that there were no matches of significance or concern.

3.4.5.2 Bioinformatic analysis for potential toxicity

The putative peptides encoded by the junction and insert sequences were compared *in silico* to the UniProt toxin protein database (TOX_2023). This database is a subset of sequences

[4] Evaluation of sequences stop-to-stop codon is a more conservative approach compared to the evaluation of start-to-stop codon sequences.
[5] http://comparedatabase.org/database/
[6] https://www.ncbi.nlm.nih.gov/protein/

derived from the Swiss-Prot protein database[7], curated to remove likely non-toxin proteins, and contained 7,227 sequences at the date of analysis (January 2023). A FASTA algorithm with a BLOSUM50 scoring matrix was used to query the putative peptides against the toxin database. No alignments with an E-score $\leq 1 \times 10^{-5}$ were identified, indicating that there was no significant homology between the putative peptides and any known protein toxins.

3.5 Conclusion

The data provided by the applicant showed that an integration event has occurred at a single locus in the soybean genome. Sequencing data confirmed that the *cry1A.2* and *cry1B.2* expression cassettes are present in the genome of MON94637 and have the expected sequence and organisation. No plasmid backbone sequences, including antibiotic resistance genes, from the transforming PV-GMIR527237 plasmid are present. The T-DNA II from PV-GMIR527237, which was inserted in the initial transformation, is not present in MON94637. The introduced DNA was shown to be stably inherited and expressed across several breeding generations of MON94637. None of the new RFs created by the insertion raise any allergenicity or toxicity concerns.

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

4 Characterisation and safety assessment of novel substances

In considering the safety of novel proteins it is important to understand that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, because of anti-nutrient properties or triggering of 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, concentration and localisation of all newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects 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 MON94637, Cry1A.2 and Cry1B.2. Both proteins are members of the crystal (Cry) family of pore-forming proteins produced by *B. thuringiensis*. Cry proteins are contact pesticides, requiring ingestion by the target pest[8] and passage into the digestive system in order to exert their effect (Jurat-Fuentes and Crickmore 2017). Alkaline conditions and proteases in the insect midgut cause the proteolytic cleavage of the Cry protein's protoxin domain and activation of the insecticidal toxin. The activated protein functions by binding to a highly specific glycoprotein receptor on the surface of midgut epithelial cells, aggregating and forming pores in the cell membrane (Schnepf et al. 1998). This leads to loss of cell integrity in the midgut, leading to developmental delays (growth inhibition) and insect death.

The high specificity of Cry proteins for particular target insects has made them a popular tool in GM crops to manage insect pests (Zwack et al. 2024). However, the emergence of resistance to these proteins has led to an ongoing need to identify and develop new insecticidal proteins with continuing efficacy against resistant insects. The diversity of the Cry family of proteins arises from three structural domains that evolved independently, and by swapping domains between toxins (de Maagd et al. 2003). Similarities in the structural domains make it possible to engineer novel chimeric proteins in the laboratory through the exchange of homologous DNA domains between different *cry* genes (Deist et al. 2014). The aim of this work is to improve the specific activity, or to broaden the spectrum of insecticidal activity of the encoded Cry protein.

4.1 Cry1A.2

Cry1A.2 is a chimeric protein designed using the domain exchange strategy with the goal to achieve high levels of activity against target insect pests of the lepidopteran order. The chimeric protein consists of three structural domains and one C-terminal protoxin domain. These four domains are from three different Cry proteins (Figure 3). Each domain shows 100% sequence similarity to the Cry protein from which it was derived. The Cry1A.2 is developed using the following cry proteins:

- Cry1Ah from *B. thuringiensis* (domain I)
- Cry1Ac from *B. thuringiensis* subsp. *kurstaki* (domain II and C-terminal protoxin domain)
- Cry1Ca from *B. thuringiensis* subsp. *aizawai* (domain III)

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

By exchanging domains, Cry1A.2 has been engineered to have enhanced specificity for southern armyworm (Chen et al. 2021). The *cry1A.2* gene prepared by the applicant encodes a protein of 1189 amino acids, with an apparent molecular weight of ~135 kDa.

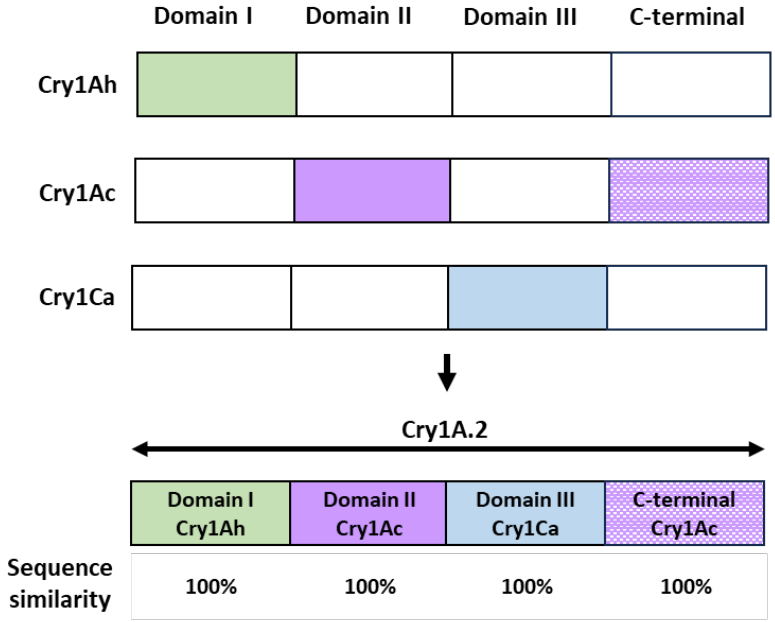

Figure 3. *Representation of the four B. thuringiensis domains from which the Cry1A.2 protein is derived.*

4.1.1 Characterisation of Cry1A.2 expressed in MON94637 and equivalence to a bacterially-produced form

The equivalence of MON94637- and *B. thuringiensis*-produced Cry1A.2 proteins must be established before the safety data generated using *B. thuringiensis*-derived Cry1A.2 can be applied to MON94637-produced Cry1A.2 protein.

Plant-produced Cry1A.2 protein was purified from MON94637 grain using a combination of anion-exchange and immunoaffinity chromatography. *B. thuringiensis*-produced Cry1A.2 protein was generated following the fermentation of *B. thuringiensis* containing a plasmid expressing the Cry1A.2 protein.

In order to confirm the identity and equivalence for the MON94637- and *B. thuringiensis*-produced Cry1A.2 proteins, a series of analytical tests were done, the results of which are summarised below.

Molecular weight analysis

Aliquots of purified MON94637- and *B. thuringiensis*-produced Cry1A.2 proteins were subjected to SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. The MON94637- and *B. thuringiensis*-produced Cry1A.2 migrated with the same apparent molecular weight of 124.2 kDa, which was within the acceptance limit for equivalence. The average purity of the MON94637-produced Cry1A.2 was determined to be 98%.

Western blot and immunoreactivity analysis

Western blot analysis with a Cry1A.2-specific antibody showed that the protein being expressed in MON94637 and *B. thuringiensis* was indeed Cry1A.2 and they have equivalent

immunoreactivity.

N-terminal sequencing

N-terminal sequencing confirmed that the first 15 amino acids of MON94637- and *B. thuringiensis*-produced Cry1A.2 proteins were as expected based on the *cry1A.2* gene.

Peptide mass fingerprint analysis

MON94637-produced and *B. thuringiensis*-produced Cry1A.2 were digested with trypsin and analysed by LC-MS/MS. For MON94637-produced Cry1A.2, 139 unique peptides were identified, covering 95% of the expected protein sequence (1135 of 1189 amino acids). For *B. thuringiensis*-produced Cry1A.2, 113 unique peptides, covering 88% of the expected protein sequence (1057 of 1189 amino acids), were identified. These results further confirm that the protein being expressed in MON94637 and *B. thuringiensis* is Cry1A.2.

Glycosylation analysis

An enhanced chemiluminescence (ECL) glycoprotein detection procedure showed the Cry1A.2 proteins from both MON94637 and *B. thuringiensis* were equivalent and that neither is glycosylated.

Functional activity analysis

The biological activity of MON94637- and *B. thuringiensis*-produced Cry1A.2 was evaluated in a 7-day insect bioassay. In this assay, European corn borer insect larvae fed diet containing MON94637- and *B. thuringiensis*-produced Cry1A.2 showed a mean EC_{50} value of 0.13 µg protein/ml diet and 0.10 µg protein/ml diet, respectively. These EC_{50} values are within acceptance limits for equivalence.

Conclusion

The results outlined in this section demonstrated that *B. thuringiensis*-produced Cry1A.2 is structurally, biochemically and functionally equivalent to MON94637-produced Cry1A.2. It can be concluded that *B. thuringiensis*-produced Cry1A.2 is a suitable surrogate for use in the safety assessment experiments described in Section 4.1.2.

4.1.2 Safety of the introduced Cry1A.2

4.1.2.1 Bioinformatic analyses of Cry1A.2

Bioinformatic analyses, as described in Section 3.4.5.1, were performed to compare the Cry1A.2 amino acid sequence to known allergenic proteins in the COMPARE allergen database (AD_2023). The search did not identify any known allergens with homology to Cry1A.2. No alignments had an E-score of $\leq 1\times10^{-5}$ 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 Cry1A.2 sequence and proteins in the allergen database.

The applicant also provided the results of *in silico* analyses comparing the amino acid sequence of Cry1A.2 to proteins identified as “toxins” in the TOX_2023 database, as described in Section 3.4.5.2. The search did not identify any known toxins with homology to Cry1A.2.

4.1.2.2 Susceptibility of Cry1A.2 to digestion with pepsin and pancreatin

B. thuringiensis-produced Cry1A.2 (test protein) was mixed with pepsin (10U enzyme/µg protein), then incubated for 0-60 min at 37°C. Reactions occurred under acidic conditions in simulated gastric fluid (Thomas et al. 2004). A control mixture lacking pepsin, and a separate control lacking Cry1A.2, 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-Cry1A.2 antibody. Concurrently, a serial dilution of the reaction mix (test protein plus enzyme) without incubation was used to determine the limit of detection (LOD) for the Cry1A.2 protein after gel staining and Western blotting. In the protein gel staining analysis, 1 µg test protein was loaded per lane and the LOD was calculated to be ~6.25 ng. In the Western blotting experiments, 10 ng test protein was loaded and the LOD was ~1.0 ng.

Visual inspection of both the stained gel and the Western blot showed that by 0.5 min of incubation, the amount of intact Cry1A.2 remaining in the reaction mix was below the LOD. Based on the LOD, it was calculated that >99.4% of the intact Cry1A.2 was digested within 0.5 min when analysed by SDS-PAGE and >90% when analysed by Western blot. Peptide fragments between ~3.5 to 6 kDa was observed in the SDS-PAGE through 60 min of digestion. No smaller peptides were detected in the Western blot at any time beyond 0.5 min. Cry1A.2 in the control mixture lacking pepsin remained intact after 60 min of incubation, indicating that the rapid loss of Cry1A.2 protein in the reaction mixes is due to proteolytic digestion of Cry1A.2.

To assess the susceptibility of Cry1A.2 to pancreatin[9] digestion, *B. thuringiensis*-produced Cry1A.2 was incubated with pancreatin (~55 µg pancreatin/µg Cry1A.2) at 37°C for 0-24 hours (h), in a simulated intestinal fluid system at a neutral pH range. A control mixture lacking pancreatin, and a separate control lacking Cry1A.2, 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 without incubation was used to determine the LOD for the protein, which was approximately 1.0 ng. For the digestibility analysis, ~10 ng of protein was loaded per lane.

Visual inspection of the Western blot showed that after 5 min of incubation, the level of intact Cry1A.2 remaining in the reaction mix was below the LOD. Based on the LOD, it was calculated that >90% of the intact Cry1A.2 protein was degraded within 5 min. After 24 h of incubation, the majority of Cry1A.2 was still intact in the control mixture lacking pancreatin, although a small amount of a 75 kDa fragment was seen observed. However, this fragment is larger than what was generated by pancreatin, indicating that the loss of Cry1A.2 in the reaction mix is indeed due to proteolytic digestion of Cry1A.2 by pancreatin.

In the human digestive system, gastric digestion (pepsin) occurs before intestinal digestion (pancreatin). Therefore a sequential digestion was performed on Cry1A.2. The pepsin digestion was run for 2 min followed by digestion by pancreatin for 0-2 h. The results showed that by 2 min, intact Cry1A.2 was digested by the pepsin and the small transiently-stable peptide fragments of ~3.5 to 6 kDa were completely digested within 0.5 min of pancreatin exposure. No other bands were present in the 0.5 min sequential digestion, other than those found in the controls.

Together, these data indicate that Cry1A.2 will be fully degraded by gastric and intestinal enzymes in the human digestive system.

[9] Pancreatin is a mixture of proteolytic enzymes

4.1.2.3 Structural stability and bioactivity of Cry1A.2 after exposure to heat

To assess stability after heating, *B. thuringiensis*-produced Cry1A.2 protein 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 run on SDS-PAGE and stained with Brilliant Blue G-Colloidal stain to detect the extent of protein degradation. No visible degradation or decrease in band intensity was observed for Cry1A.2 in the control, 25 and 37°C treated samples at both 15 and 30 min. At 55°C, aggregation products were observed at both 15 and 30 min. At 75°C, degradation and aggregation products were present and a more prominent loss of band intensity of Cry1A.2 at 30 min was observed. The samples treated at 95°C for 15 and 30 min had a significant loss of Cry1A.2 band intensity. Relative to the 75°C samples, an increase in degradation products was observe at 95°C for 15 and 30 min. These data indicate that Cry1A.2 is heat labile at temperatures greater than 75°C

To assess the functionality (bioactivity) of Cry1A.2 after heating, heated protein samples were tested in a bioassay using soybean looper (SBL) larvae (*Chrysodeixi includens*). Control insects were fed a diet containing unheated Cry1A.2 which had been maintained on ice (~0°C). The bioactivity for each sample was measured as a 7-day EC_{50} value, which is the Cry1A.2 concentration that results in a 50% growth inhibition relative to control insects. At heating temperatures of 25, 37 or 55°C for either 15 or 30 min, the activity of Cry1A.2 remained similar to the unheated Cry1A.2 with a slight reduction in protein activity at the 55°C for 30 min heat treatment (Table 4). At 75 or 95°C, no dose-response relationship to the samples was observed, indicating that Cry1A.2 activity was reduced to the extent than an EC50 value could not be established (<50% growth inhibition at the highest protein concentration tested). These results indicate that heating to temperatures of 75°C or above effectively destroys the functional activity of Cry1A.2.

Table 4: Bioactivity of heat-treated Cry1A.2 in a diet fed to insect larvae

Temperature	15 min heat treatment		30 min heat treatment	
	EC_{50} (µg Cry1A.2/ml diet)	**95% CI[b]** (µg Cry1A.2/ml diet)	**EC_{50}** (µg Cry1A.2/ml diet)	**95% CI[b]** (µg Cry1A.2/ml diet)
0°C (control)	0.058	0.054 – 0.064	0.058	0.054 – 0.064
25°C	0.079	0.070 – 0.090	0.092	0.070 – 0.090
37°C	0.071	0.063 – 0.081	0.064	0.063 – 0.081
55°C	0.073	0.065 – 0.081	0.190	0.065 – 0.081
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. EC50 value 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 MON94637-produced Cry1A.2 protein confirming its identity and functionality as well as equivalence to the corresponding protein produced in a bacterial expression system. Bioinformatic analyses showed Cry1A.2 had no amino acid sequence similarity to known toxins or allergens. The protein was shown to be

inactivated by heating and susceptible to pepsin and pancreatin digestion. Taken together this indicates that the Cry1A.2 protein is unlikely to be toxic or allergenic to humans.

4.2 Cry1B.2

Cry1B.2 is a chimeric protein designed using the domain exchange strategy with the goal to achieve high levels of activity against target insect pests of the lepidopteran order. The chimeric protein consist of three structural domains and one C-terminal protoxin domain. These four domains are from three different Cry proteins (Figure 4). Each domain shows 100% sequence similarity to the Cry protein from which it was derived. The Cry1B.2 is developed using the following cry proteins:

- Cry1Be from *B. thuringiensis* (domain I and II)
- Cry1Ka2 from *B. thuringiensis* subsp. *morrisoni* (domain III)
- Cry1Ab from *B. thuringiensis* subsp. *kurstaki* (C-terminal protoxin domain)

By exchanging domains, Cry1B.2 has been engineered to have enhanced specificity for fall armyworm (Chen et al. 2021). The *cry1A.2* gene prepared by the applicant encodes a protein of 1187 amino acids, with an apparent molecular weight of ~135 kDa.

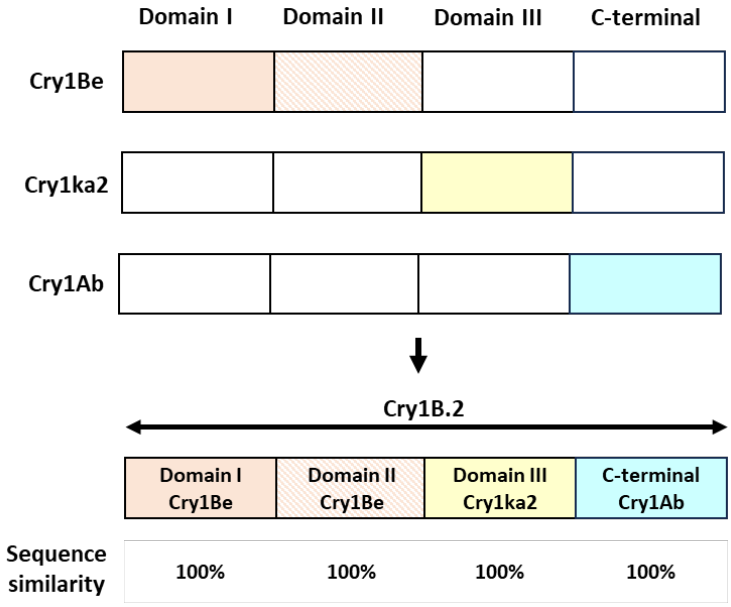

Figure 4. *Representation of the four B. thuringiensis domains from which the Cry1B.2 protein is derived.*

4.2.1 Characterisation of Cry1B.2 expressed in MON94637 and equivalence to a bacterially-produced form

The equivalence of MON94637- and *B. thuringiensis*-produced Cry1B.2 proteins must be established before the safety data generated using *B. thuringiensis*-derived Cry1B.2 can be applied to MON94637-produced Cry1B.2 protein.

The plant-produced Cry1B.2 protein was purified from MON94637 grain using a combination of anion-exchange and immunoaffinity chromatography. *B. thuringiensis*-produced Cry1B.2 protein was generated following the fermentation of *B. thuringiensis* containing a plasmid expressing the protein.

In order to confirm the identity and equivalence for the MON94637- and *B. thuringiensis*-produced Cry1B.2 proteins, a series of analytical tests were done, the results of which are summarised below.

Molecular weight analysis

Aliquots of purified MON94637- and *B. thuringiensis*-produced Cry1B.2 proteins were subjected to SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. The MON94637- and *B. thuringiensis*-produced Cry1B.2 migrated with the same apparent molecular weights of 135.5 kDa, which was within the acceptance limit for equivalence. The average purity of the MON94637-produced Cry1B.2 was determined to be 77%.

Western blot and immunoreactivity analysis

Western blot analysis with an Cry1B.2-specific antibody showed that the protein being expressed in MON94637 and *B. thuringiensis* was indeed Cry1B.2 and they have equivalent immunoreactivity.

N-terminal sequencing

N-terminal sequencing confirmed that the first 15 amino acids of MON94637- and *B. thuringiensis*-produced Cry1B.2 proteins were as expected based on the *cry1B.2* gene.

Peptide mass fingerprint analysis

MON94637-produced and *B. thuringiensis*-produced Cry1B.2 were digested with trypsin and analysed by LC-MS/MS. For MON94637-produced Cry1B.2, 115 unique peptides were identified, covering 92% of the expected protein sequence (1097 of 1187 amino acids). For *B. thuringiensis*-produced Cry1B.2, 89 unique peptides, covering 66% of the expected protein sequence (792 of 1187 amino acids), were identified. These results further confirm that the protein being expressed in MON94637 and *B. thuringiensis* is Cry1B.2.

Glycosylation analysis

An ECL glycoprotein detection procedure showed the Cry1B.2 proteins from both MON94637 and *B. thuringiensis* were equivalent and that neither is glycosylated.

Functional activity analysis

The biological activity of MON94637- and *B. thuringiensis*-produced Cry1B.2 was evaluated in a 7-day insect bioassay. In this assay, European corn borer insect larvae fed diet containing MON94637- and *B. thuringiensis*-produced Cry1B.2 showed a mean EC_{50} value of 0.61 µg protein/ml diet and 0.71 µg protein/ml diet, respectively. These EC_{50} values are within acceptance limits for equivalence.

Conclusion

The results outlined in this section demonstrated that *B. thuringiensis*-produced Cry1B.2 is structurally, biochemically and functionally equivalent to MON94637-derived Cry1B.2. It can be concluded that *B. thuringiensis*-produced Cry1B.2 is a suitable surrogate for use in the safety assessment experiments described in Section 4.2.2.

4.2.2 Safety of the introduced Cry1B.2

4.2.2.1 Bioinformatic analyses of Cry1B.2

Bioinformatic analyses, as described in Section 3.4.5.1, were performed to compare the Cry1B.2 amino acid sequence to known allergenic proteins in the COMPARE allergen database (AD_2023). The search did not identify any known allergens with homology to Cry1B.2. No alignments had an E-score of $\leq 1\times10^{-5}$ 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 Cry1B.2 sequence and proteins in the allergen database.

The applicant also provided the results of *in silico* analyses comparing the amino acid sequence of Cry1B.2 to proteins identified as “toxins” in the TOX_2023 database, as described in Section 3.4.5.2. The search did not identify any known toxins with homology to Cry1B.2.

4.2.2.2 Susceptibility of Cry1B.2 to digestion with pepsin and pancreatin

B. thuringiensis-produced Cry1B.2 (test protein) was mixed with pepsin (10U enzyme/µg protein), then incubated for 0-60 min at 37°C. Reactions occurred under acidic conditions in simulated gastric fluid (Thomas et al. 2004). A control mixture lacking pepsin, and a separate control lacking Cry1B.2, 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-Cry1B.2 antibody. Concurrently, a serial dilution of the reaction mix (test protein plus enzyme) without incubation was used to determine the limit of detection (LOD) for the Cry1B.2 protein after gel staining and Western blotting. In the protein gel staining analysis, 1 µg test protein was loaded per lane and the LOD was calculated to be ~6.25 ng. In the Western blotting experiments, 10 ng test protein was loaded and the LOD was ~1.25 ng.

Visual inspection of both the stained gel and the Western blot showed that by 0.5 min of incubation, the amount of intact Cry1B.2 remaining in the reaction mix was below the LOD. Based on the LOD, it was calculated that >99.4% of the intact Cry1B.2 was digested within 0.5 min when analysed by SDS-PAGE and >87.5% when analysed by Western blot. Peptide fragments ~4 kDa were observed in the SDS-PAGE through 60 min of digestion. No smaller peptides were detected in the Western blot at any time beyond 0.5 min. Cry1B.2 in the control mixture lacking pepsin remained intact after 60 min of incubation, indicating that the rapid loss of Cry1B.2 protein in the reaction mixes is due to proteolytic digestion of Cry1B.2.

To assess the susceptibility of Cry1B.2 to pancreatin digestion, *B. thuringiensis*-produced Cry1B.2 was incubated with pancreatin (~55 µg pancreatin/µg Cry1A.2) at 37°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 Cry1B.2, 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 without incubation was used to determine the LOD for the protein, which was approximately 1.25 ng. For the digestibility analysis, ~10 ng of protein was loaded per lane.

Visual inspection of the Western blot showed that after 5 min of incubation, the level of intact Cry1B.2 remaining in the reaction mix was below the LOD. Based on the LOD, it was calculated that >87.5% of the intact Cry1B.2 protein was degraded within 5 min. The Cry1B.2 in the control mixture lacking pancreatin was truncated to ~75 kDa fragment after 24 h of incubation. However, this fragment is larger than what was generated by pancreatin, indicating that that the loss of Cry1B.2 in the reaction mix is indeed due to proteolytic

digestion of Cry1B.2 by pancreatin.

A sequential digestion was performed on Cry1B.2. The pepsin digestion was run for 2 min followed by digestion by pancreatin for 0-2 h. The results showed that by 2 min, intact Cry1B.2 was digested by the pepsin and the small transiently-stable peptide fragments of ~4 kDa were completely digested within 0.5 min of pancreatin exposure. No other bands were present in the 0.5 min sequential digestion, other than those found in the controls.

Together, these data indicate that Cry1B.2 will be fully degraded by gastric and intestinal enzymes in the human digestive system.

4.2.2.3 Structural stability and bioactivity of Cry1B.2 after exposure to heat

To assess stability after heating, *B. thuringiensis*-produced Cry1B.2 protein 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 run on SDS-PAGE and stained with Brilliant Blue G-Colloidal stain to detect the extent of protein degradation. No visible degradation or decrease in band intensity was observed for Cry1B.2 in the control, 25, 37 or 55°C treated samples at both 15 and 30 min. At 75°C, degradation and aggregation products were present and a more prominent loss of band intensity of Cry1B.2 at 30 min was observed. The samples treated at 95°C for 15 and 30 min had a significant loss of Cry1B.2 band intensity. Relative to the 75°C samples, an increase in aggregation and degradation products was observe at 95°C for 15 and 30 min. These data indicate that Cry1B.2 is heat labile at temperatures greater than 75°C.

To assess the functionality (bioactivity) of Cry1B.2 after heating, heated protein samples were tested in a bioassay using SBL larvae. Control insects were fed a diet containing unheated Cry1B.2 which had been maintained on ice (~0°C). The bioactivity for each sample was measured as a 7-day EC_{50} value, which is the Cry1B.2 concentration that results in a 50% growth inhibition relative to control insects. At heating temperatures of 25, 37 or 55°C for either 15 or 30 min, the activity of Cry1A.2 remained similar to the unheated Cry1B.2 (Table 5). At 75 or 95°C, no dose-response relationship to the samples was observed, indicating that Cry1B.2 activity was reduced to the extent than 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 75°C or above effectively destroys the functional activity of at elevated temperatures Cry1B.2.

Table 5: Bioactivity of heat-treated Cry1B.2 in a diet fed to insect larvae

Temperature	15 min heat treatment		30 min heat treatment	
	EC_{50} (µg Cry1B.2/ml diet)	**95% CI[b]** (µg Cry1B.2/ml diet)	**EC_{50}** (µg Cry1B.2/ml diet)	**95% CI[b]** (µg Cry1B.2/ml diet)
0°C (control)	9.3	7.0 - 12	9.3	7.0 - 12
25°C	8.3	6.8 – 10	9.5	8.2 – 11
37°C	7.8	7.0 – 8.7	8.1	7.5 – 8.8
55°C	8.0	6.9 – 9.3	8.6	7.1 – 10
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. EC50 value could not be calculated as <50% growth inhibition was observed at the highest protein concentration tested.
b. Confidence limits

4.2.3 Conclusion

A range of characterisation studies were performed on MON94637-produced Cry1B.2 protein confirming its identity and functionality as well as equivalence to the corresponding protein produced in a bacterial expression system. Bioinformatic analyses showed Cry1B.2 had no amino acid sequence similarity to known toxins or allergens. The protein was shown to be inactivated by heating and susceptible to pepsin and pancreatin digestion. Taken together this indicates that the Cry1B.2 protein is unlikely to be toxic or allergenic to humans.

4.3 Expression levels of novel proteins

For analysis of the expression levels of the Cry1A.2 and Cry1B.2 proteins in MON94637, tissues were collected from four replicate plots at each of the five field-trial sites in representative soybean-producing regions of the United States during the 2021 growing season.[10]. Forage, grain, leaf, flower and root tissue samples were collected from each plot at specified growth stages. See Figure 5 for a summary of soybean growth stages and the stage at which each tissue type was collected.

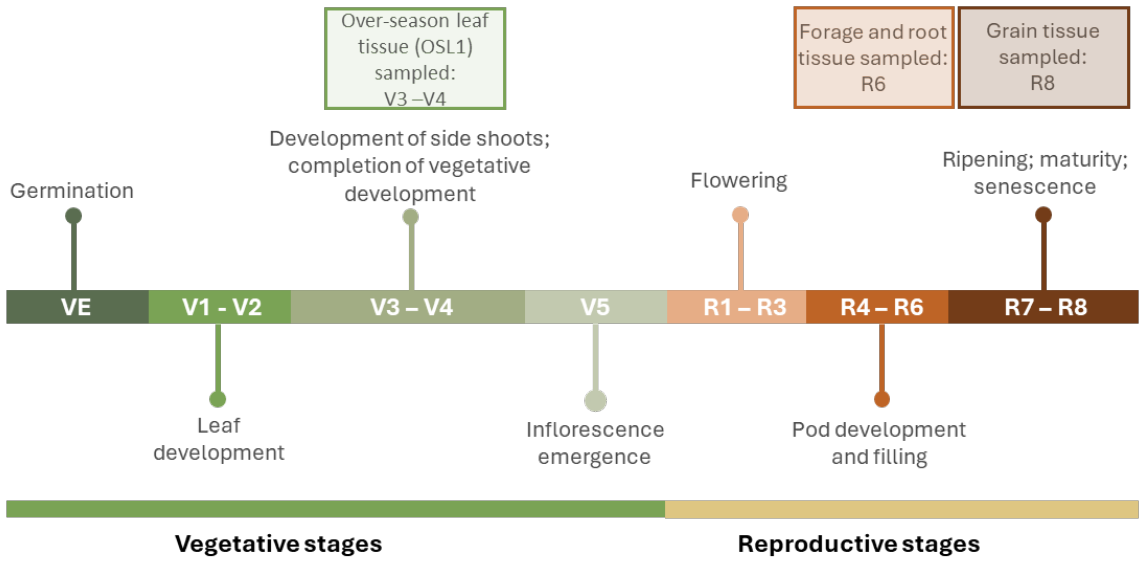

Figure 5. *Growth stages of soybean. The stages at which the leaf, flower, forage, root and grain tissue for protein expression analysis were sampled are indicated.*

Cry1A.2 and Cry1B.2 were extracted from tissues using standard methods and their expression levels were quantified in each tissue using a quantitative enzyme-linked immunosorbent assay (ELISA). The *B. thuringiensis*-produced versions of each protein were used as analytical references for the respective plant-derived proteins.

The mean level of each protein in each tissue type determined by ELISA is shown in Figure 6. Cry1A.2 had the highest mean expression levels in flower at 260 µg/g dry weight (dw) and the lowest in root at 9.8 µg/g dw. Cry1B.2 had the highest mean expression levels in leaf at 420 µg/g dw and lowest in root where the mean expression level was below the limit of

[10] Field sites for testing protein expression levels were in the following states – Illinois (2 sites), Ohio, Michigan and Nebraska.

quantification (LOQ).

For the full set of expression data, including standard errors and ranges, refer to the Application dossier[11] (pages 90 – 91).

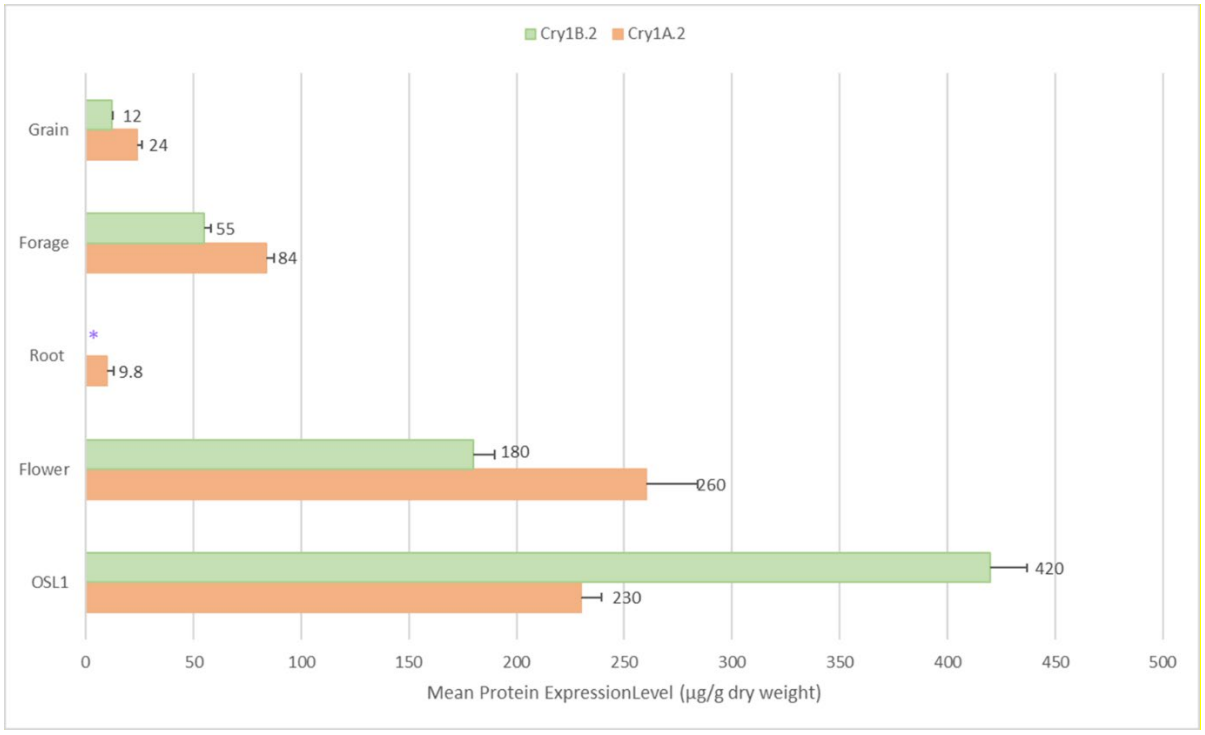

Figure 6. *Mean expression levels of the Cry1A.2 and Cry1B.2 proteins in five tissue types from MON94637. The asterisk denotes that the level of Cry1B.2 in root tissue was below the LOQ (0.313 µg/g dw).*

[11] https://www.foodstandards.gov.au/food-standards-code/applications/a1310-food-derived-insect-protected-soybean-line-mon94637

5 Compositional analysis

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

5.1 Key components

The key components to be analysed for the comparison of GM and conventional soybean are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of soybean (OECD 2012), and include: proximates, fibre, amino acids, fatty acids, minerals, vitamins, anti-nutrients and isoflavones.

5.2 Study design

MON94637 (R6 generation) and a non-GM control of similar genetic background (A3555) were grown and harvested from five field trial sites in the United States during the 2021 growing season.[12] The sites were representative of soybean 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 growing region.

At maturity, grain was harvested from all plots, ground and stored at -20°C before being shipped to an analytical laboratory on dry ice. Samples were stored at -20°C until analysis. Compositional 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 analysis, or other published scientific methods.

58 different analytes were measured in grain (see Figure 7 for a complete list). In addition, moisture was also measured and used to convert the analyte values from fresh to dry weight, but was not analysed statistically. Analytes were expressed as either percent dry weight (% dw), µg/g dw or as a percentage of total fatty acids (% total FA), as shown in Figure 13. Of the 58 analytes measured, 11 had more than 50% of observations below the LOQ (listed in grey in Figure 7) and were excluded from the statistical analyses, leaving a total of 47 components that were fully analysed in grain.

A linear mixed model analysis of variance was applied on data combined across the five

[12] The states in which the five field trial sites were located: Illinois (2 sites), Ohio, Michigan and Nebraska.

replicated field trial sites. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, North Carolina 2012). For each analyte, 'descriptive statistics' (mean, standard error (SE), and range) were generated.

In assessing the statistical significance of any difference between MON94637 and the conventional control, a *p*-value of 0.05 was used. Any statistically significant differences were evaluated further to assess whether they were likely to be biologically meaningful. The magnitude of differences in mean values between MON94637 and the control were determined, and this difference was compared to the variation observed within the control grown at multiple sites. A mean difference less than the variability seen due to natural environmental variation within the single, closely related germplasm is typically not a food or feed safety concern (Venkatesh et al. 2014).

In addition, the natural variation of analytes from the literature and from the AFSI Crop Composition Database (AFSI CCDB) was also considered (Lundry et al. 2008; Berman et al. 2009; Bellaloui et al. 2011; Breeze et al. 2015; Thompson et al. 2016; Codex 2021; AFSI 2024). The ranges derived from these values account for variability present in non-GM soybean varieties due to a wider range of agronomic and environment conditions, as well as different genetic backgrounds.

Key analyte levels were also analysed in forage but the results are not included in this report. It is noted however that, in the combined site analysis, two of the analyte levels in MON94637 differed significantly from those of the control. However, the MON94637 mean for each of these components was within the control range value, indicating that MON94637 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. In addition, the observed MON94637 means fall within the natural variability seen in the publicly available data ranges. The differences reported for the two analytes in forage are therefore consistent with the normal biological variability that exists in soybean.

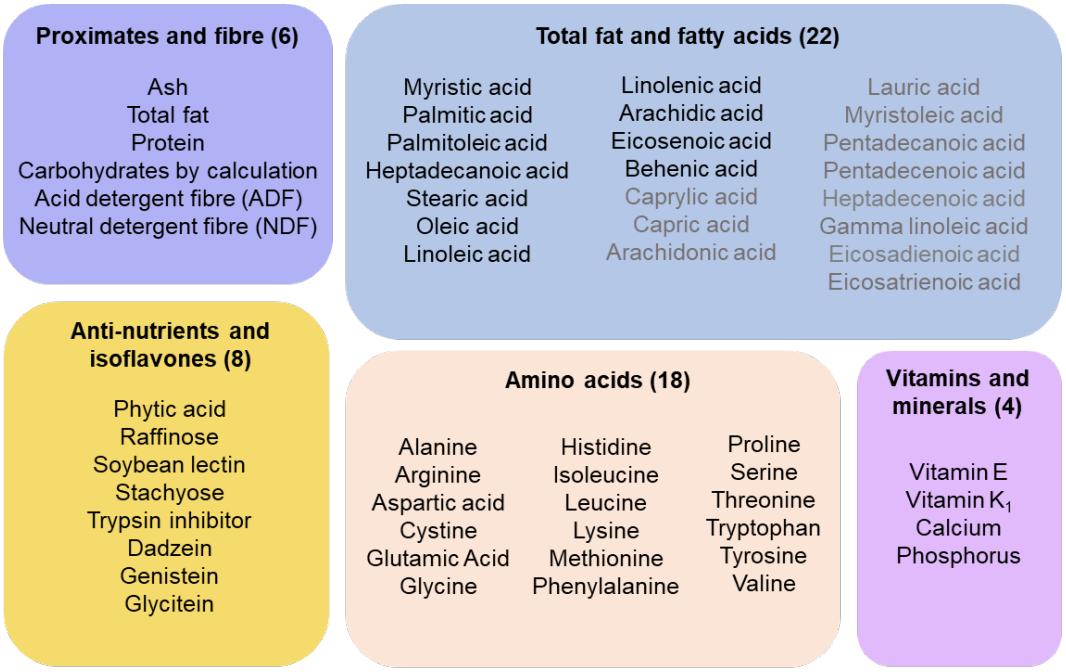

Figure 7. *Analytes measured in MON94637 grain samples. Analytes listed in grey text had >50% of samples below the LOQ and were excluded from statistical analysis. The 47 analytes listed in black text were analysed fully.*

5.3 Analyses of key components in grains

Of the 47 analytes for which mean values were provided (Figure 7), there were 3 for which there was a statistically significant difference ($p < 0.05$) between MON94637 and the control: palmitoleic acid, heptadecanoic acid and behenic acid. A summary of these 3 analytes is provided in Figure 8. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the Application dossier[13] (pages 127 – 138).

For all 3 analytes where a statistically significant difference was found, the deviation of the MON94637 mean from the control mean was less than 15% (Figure 8a). As can be observed in Figure 8 (panels b-d), the MON94637 mean for each of these analytes was within the control range value, indicating that MON94637 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. In addition, the observed MON94637 means fall within the natural variability seen in the publicly available data ranges (grey bars, Figure 8, b-d). The differences reported here are therefore consistent with the normal biological variability that exists in soybean.

Overall, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in MON94637 when compared with conventional non-GM soybean varieties already available in agricultural markets. Grain from MON94637 can therefore be regarded as equivalent in composition to grain from conventional non-GM soybean.

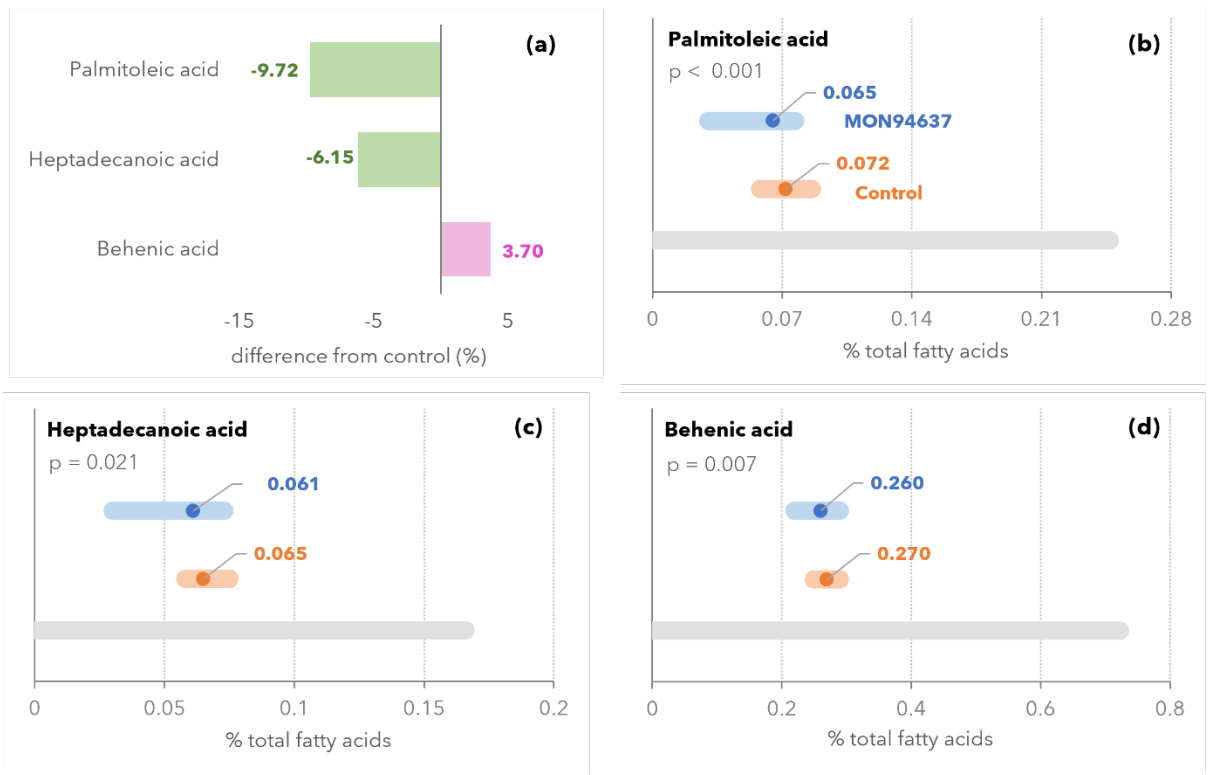

Figure 8. *Visual summary of statistically significantly analyte differences in MON94637 compared to the conventional control.* ***(a)*** *Percentage deviation of the mean MON94637 value from the mean control value for each of the 3 analytes for which significant differences were found.* ***(b) - (d)*** *Measured means (dots) and ranges for MON94637 (blue bars) and the control (orange bars) for the 3 analytes as labelled. The grey bars represent the publicly-*

[13] https://www.foodstandards.gov.au/food-standards-code/applications/a1310-food-derived-insect-protected-soybean-line-mon94637

available ranges for each analyte. Note that the x-axes vary in scale and unit for each analyte.

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 of this report.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, 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.

MON94637 is the result of genetic modifications for protection against insect pests, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modifications have not altered the nutrient composition of MON94637 compared with that of conventional non-GM soybean varieties. The introduction of food derived from MON94637 into the food supply is therefore expected to have negligible nutritional impact.

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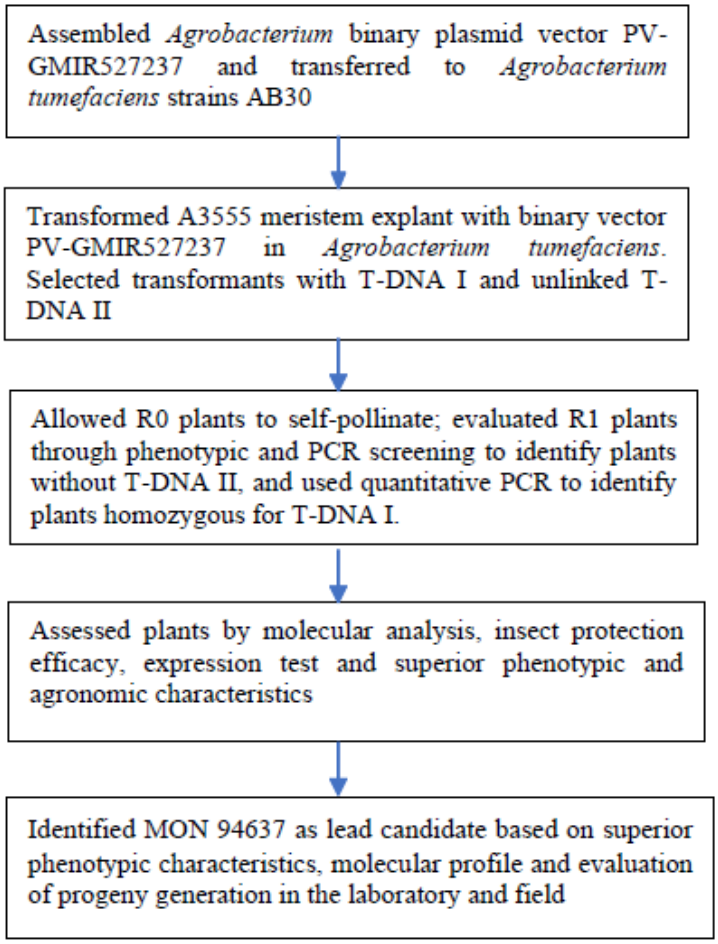
Appendix 1

Development of MON94637



Appendix 2

Genetic elements present in PV-GMHT527237

Genetic element	Relative position	Description, Source & Reference
T-DNA I		
Right Border Region	1-285	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used to transfer the T–DNA (Depicker et al. 1982; Zambryski et al. 1982)
Intervening Sequence	286-398	DNA sequence used for cloning
***cry1A.2* cassette**		
P-*ubq10-At1*	399-1600	Promoter, leader and intron from the polyubiquitin gene *ubq10* of *Arabidopsis thaliana* (Norris et al. 1993), which directs transcription in plant cells
Intervening Sequence	1601-1609	Sequence used in DNA cloning
cry1A.2	1610-5179	Codon optimized gene fusion comprised of sequences encoding Cry1Ah, Cry1Ac, and Cry1Ca domains from *Bacillus thuringiensis*, which confers protection against lepidopteran insects (Chen et al. 2021)
Intervening Sequence	5180-5187	Sequence used in DNA cloning
T-*Zfp-Mt1*	2579-3078	3' UTR sequence from *Medicago truncatula* (barrel medic) of a gene encoding a putative zinc finger protein, which directs polyadenylation of the mRNA (Hunt 1994)
Intervening Sequence	5788-5889	DNA sequence used for cloning
***Cry1B.2* cassette**		
P-*Cab-Cm1*	5890-7890	Promoter and leader of a chlorophyll a/b-binding (CAB) protein from *Cucumis melo* (melon), which directs transcription in plant cells (Hernandez-Garcia and Finer 2014).
cry1B.2	7894-11457	Codon optimized gene fusion comprised of sequences encoding Cry1Be, Cry1Ka2 and Cry1Ab domains from *B. thuringiensis*, which confers protection against lepidopteran insects (Chen et al. 2021).
T-*Lox-Mt1*	11458-11957	3' UTR sequence from a lipoxygnase gene of *M. truncatula* which directs polyadenylation of the mRNA (Hunt 1994)
Intervening Sequence	11958-12187	DNA sequence used for cloning
Left Border Region	12188-12629	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used to transfer the T–DNA (Barker et al. 1983)
Vector backbone		
Intervening Sequence	12630-12666	DNA sequence used for cloning
ble1	12667-12818	Partial coding sequence of the bleomycin resistance gene from transposon Tn5 that confers antibiotic resistance (Mazodier et al. 1985)
Intervening Sequence	12819-12838	Sequence used in DNA cloning
nptII	12839-13633	Coding sequence of the *neo* gene from transposon Tn5 of *E. coli* encoding neomycin phosphotransferase II (NPT II) (Beck et al. 1982) that confers neomycin and kanamycin resistance (Fraley et al. 1983)
P-*rrn*	13634-13858	Promoter of the ribosomal RNA operon from *Agrobacterium tumefaciens* (Bautista-Zapanta et al. 2002) that drives transcription in plant cells

Genetic element	Relative position	Description, Source & Reference
Intervening Sequence	13859-13934	Sequence used in DNA cloning
OR-*ori-pBR322*	13935-14523	Origin of replication from plasmid pBR322 for plasmid maintenance in *E. coli* (Sutcliffe 1979)
Intervening Sequence	14524-14950	Sequence used in DNA cloning
rop	14951-15142	Coding sequence of the repressor of primer protein from the ColE1 plasmid. Used for the maintenance of plasmid copy number in *E. coli* (Giza and Huang 1989)
Intervening Sequence	15143-15330	Sequence used in DNA cloning
OR-*ori-pRi*	15331-19444	Origin of replication from plasmid pRi for the maintenance of the plasmid in *Agrobacterium* (Ye et al. 2011)
Intervening Sequence	19445-19451	Sequence used in DNA cloning
T-DNA II		
Left Border Region	19452-19770	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used to transfer the T-DNA (Barker et al. 1983)
Intervening Sequence	19771-19802	Sequence used in DNA cloning
splA cassette		
T-*nos*	19803-20055	3' UTR sequence of the *nopaline synthase (nos)* gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan et al. 1983; Fraley et al. 1983)
Intervening Sequence	20056-20071	Sequence used in DNA cloning
splA	20072-21529	Coding sequence of the *splA* gene from *Agrobacterium tumefaciens* strain C58 encoding the sucrose phosphorylase protein that catalyzes the conversion of sucrose to fructose and glucose–1–phosphate (Piper et al. 1999)
Intervening Sequence	21530-21541	Sequence used in DNA cloning
P-*Usp*	21542-22720	5' UTR, promoter, and enhancer sequence of an unknown seed protein gene from *Vicia faba* (broad bean) encoding an unknown seed protein that is involved in regulating gene expression (Bäumlein et al. 1991)
Intervening Sequence	22721-22771	DNA sequence used for cloning
aadA cassette		
T-*E9*	22772-23414	3' UTR sequence from *Pisum sativum* (pea) *rbcS* gene family encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi et al. 1984) that directs polyadenylation of the mRNA
Intervening Sequence	23415-23429	Sequence used in DNA cloning
aadA	23430-24221	Coding sequence for an aminoglycoside modifying enzyme, 3'(9)-*O*-nucleotidyltransferase, from the transposon Tn7 (Fling et al. 1985) that confers spectinomycin and streptomycin resistance
TS-*CTP2*	24222-24449	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Klee et al. 1987; Herrmann 1995)
Intervening Sequence	24450-24458	Sequence used in DNA cloning
P-*EF-1*α	24459-25606	Promoter, leader, and intron sequences of the *EF–1*α gene from *Arabidopsis thaliana* encoding elongation factor EF–1α (Axelos et al. 1989) that directs transcription in plant cells
Intervening Sequence	25607-25629	Sequence used in DNA cloning

Genetic element	Relative position	Description, Source & Reference
E-*FMV*	25630-26166	Enhancer from the 35S RNA of figwort mosaic virus (FMV) (Richins et al. 1987) that enhances transcription in most plant cells (Rogers 2000)
Intervening Sequence	26167-26212	Sequence used in DNA cloning
B-Right Border Region	26213-26543	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used to transfer the T–DNA (Depicker et al. 1982; Zambryski et al. 1982)
Vector backbone		
Intervening Sequence	24535-24549	Sequence used in DNA cloning