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

Supporting document 1

Safety Assessment Report (at Approval) –Application A1097

Food derived from Herbicide-tolerant & Insect-protected Corn Line MON87411

Summary and conclusions

Background

A genetically modified (GM) corn line with OECD Unique Identifier MON-87411-9, hereafter referred to as MON87411, has been developed by Monsanto Company (Monsanto).

MON87411 contains three expression cassettes:

- A *DvSnf7* cassette contains two *DvSnf7* fragments in an inverted repeat sequence. Expression of the cassette results in the formation of a double-stranded RNA (dsRNA) transcript. When the dsRNA is ingested by the corn rootworm (CRW) insect, it triggers RNA interference (RNAi) of the CRW *DvSnf7* gene, leading to death of the insect.
- A *cry3Bb1* cassette contains the *cry3Bb1* gene that confers tolerance to CRW via the expression of the Cry3Bb1 protein which has a direct adverse effect on the gut of insects feeding on the plant.
- A *cp4 epsps* cassette contains the *cp4 epsps* gene that encodes a protein conferring tolerance to the herbicide glyphosate.

In conducting a safety assessment of food derived from MON87411, a number of criteria have been addressed including: a characterisation of the transferred gene sequences, their origin, function and stability in the corn genome; the changes at the level of DNA, protein and RNA in the whole food; compositional analyses; and evaluation of intended and unintended changes.

This safety assessment report addresses only food safety and nutritional issues. It therefore does not address:

- environmental risks related to the environmental release of GM plants used in food production
- the safety of animal feed, or animals fed with feed, derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of Use

In terms of production, corn is the world's dominant cereal crop, ahead of wheat and rice and is grown in over 160 countries. It has a long history of safe use in the food supply. Sweet corn is consumed directly while 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). Corn is also widely used as a feed for domestic livestock.

Molecular Characterisation

MON87411 was generated through *Agrobacterium*-mediated transformation. Comprehensive molecular analyses of MON87411 indicate there is a single insertion site comprising a single, complete copy of each of the three expression cassettes. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and directed sequencing analysis shows no plasmid backbone has been incorporated into the transgenic locus.

Northern blot analysis of RNA extracted from MON87411 confirmed that a primary single-stranded RNA transcript of approximately <1.2 kb (comprising 968 nucleotides of DvSnf7 sequence, poly-A tail and 5' cap) is produced in the plant. Northern blot analysis also confirmed the primary transcript forms into a 240 bp dsRNA, which is the expected size.

Characterisation and safety assessment of new substances

Newly expressed proteins

Corn line MON87411 expresses two novel proteins, Cry3Bb1 and CP4 EPSPS. For both proteins, mean levels were lowest in the grain (4.0 and 1.9 µg/g dry weight, respectively). The highest levels of both proteins were in the whole plant samples at the V3 – V4 stage (340 and 63 µg/g dry weight, respectively) as would be expected from the high levels in both the leaves and roots at this stage.

A range of characterisation studies confirmed the identity of the Cry3Bb1 and CP4 EPSPS proteins produced in MON87411. The plant Cry3Bb1 and CP4 EPSPS proteins have the expected molecular weight (approximately 77 kDa and 43 kDa respectively), immunoreactivity, lack of glycosylation, amino acid sequence and enzyme activity.

There are no concerns regarding the potential toxicity or allergenicity of the expressed proteins. Previous assessments of both Cry3Bb1 and CP4 EPSPS have confirmed their safety. Additionally, updated bioinformatic studies assessed as part of this application have confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens.

ds DvSnf RNA

In addition to two novel proteins, corn line MON87411 also expresses a 240 bp dsRNA containing sequences from the *DvSnf7* gene. The 240 bp dsRNA is detectable in a variety of tissues in MON87411, its highest expression being in whole plants around 22 days after planting (0.085 µg/g dw) and its lowest expression in the grain (0.104 ng/g dw).

A number of studies have been done to confirm the mechanism of action of the DvSnf7 dsRNA produced in MON87411. These studies show that once ingested by the insect, the 240 bb dsRNA is taken up by the cells of the insect midgut and subsequently processed by the insect's RNAi machinery into 21-24-mer small interfering RNAs (siRNAs).

These dsRNA and/or siRNAs are able to spread systemically throughout the insect and act to down regulate the *DvSnf7* gene, subsequently leading to the death of the insect.

There are no concerns regarding the safety of the DvSnf7 dsRNA in MON87411. The data provided 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 these RNAs 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. In addition, the level of the DvSnf7 dsRNA present in grain from MON87411 is extremely low, and the anti-*DvSnf7* effect observed in corn rootworm is also highly specific to only a very small number of closely-related beetles. Grain containing the DvSnf7 dsRNA is therefore considered to be as safe for human consumption as grain derived from conventional corn varieties.

Herbicide Metabolites

The herbicide residues resulting from the application of glyphosate to lines carrying the *cp4 epsps* gene have been previously assessed by FSANZ. There are no concerns that the spraying of MON87411 with glyphosate would result in the production of any novel metabolites that have not been previously considered.

Compositional Analyses

Detailed compositional analyses established the nutritional adequacy of grain from MON87411 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients. Only 11 of the 52 reported analytes deviated in level from the control in a statistically significant manner. However, the mean levels of all of these analytes were consistent with natural variation. It can therefore be concluded that grain from MON87411 is compositionally equivalent to grain from conventional corn varieties.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant and insect-protected corn line MON87411-9. On the basis of the data provided in the present Application, and other available information, food derived from MON87411 is considered to be as safe for human consumption as food derived from conventional corn varieties.

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List of Abbreviations

ADF	acid detergent fibre
BLAST	Basic Local Alignment Search Tool
bp	base pairs
Bt	Bacillus thuringiensis
CPB	Colorado potato beetle
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4
CRW	corn rootworm
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
dsRNA	double-stranded RNA
dw	dry weight
ELISA	enzyme linked immunosorbent assay
ESCRT	Endosomal Sorting Complex Required for Transport
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
FSANZ	Food Standards Australia New Zealand
GM	genetically modified
IgE	Immunoglobulin E
JSA	junction sequence analysis
kDa	kilo Dalton
LB	Left Border of T-DNA
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI-TOF MS	matrix-assisted laser desorption/ionisation–time of flight mass spectrometry
NDF	neutral detergent fibre
NGS	next generation sequencing
nt	nucleotide
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PCR	polymerase chain reaction
P-value	probability value
RB	Right Border of T-DNA
RNA	ribonucleic acid
RNAi	RNA interference
miRNA	micro RNA
siRNA	small interfering RNA
SAS	Statistical Analysis Software
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
U.S.	United States of America

1 Introduction

Monsanto Australia Limited has submitted an application to FSANZ to vary Standard 1.5.2 – Food produced using Gene Technology – in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) corn line MON-87411-9 (referred to as MON87411). The corn has been modified such that it is both tolerant to the herbicide glyphosate and protected against corn rootworm (CRW) (*Diabrotica* spp.), an insect pest.

Tolerance to glyphosate is achieved through expression of the enzyme 5-enolpyruvyl-3-shikimatephosphate synthase (CP4 EPSPS) encoded by the *cp4 epsps* gene derived from the common soil bacterium *Agrobacterium* sp. strain CP4. The safety of the CP4 EPSPS protein has previously been assessed by FSANZ.

Protection against CRW occurs via two genetic modifications:

- The expression of a *cry3Bb1* gene that produces a modified *Bacillus thuringiensis* (subsp. *kumamotoensis*) Cry3Bb1 protein to protect against CRW larval feeding. The safety of the Cry3Bb1 protein has previously been assessed by FSANZ.
- The expression of a suppression cassette containing an inverted repeat sequence that corresponds to a region of the *DvSnf7* gene from western corn rootworm (*Diabrotica virgifera virgifera*). Expression of the inverted repeat results in the formation of a double-stranded RNA (dsRNA) transcript containing a fragment of the *DvSnf7* gene. When ingested by CRW, it is recognised by the insect's RNA interference (RNAi) machinery leading to the down regulation of the CRW *DvSnf7* gene and ultimately death of the insect.

According to the Applicant, MON87411 will not be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding, with other approved GM corn lines (a process known as 'stacking'). It is further intended that any lines containing the MON87411 event will be grown in North America, and approval for cultivation in Australia or New Zealand is not being sought. Therefore, if approved, food derived from this line may enter the Australian and New Zealand food supply as imported food products.

2 History of use

2.1 Host organism

Mature corn (*Zea mays*) plants contain both female and male flowers and usually reproduce sexually by wind-pollination. This provides for both self-pollination and natural out-crossing between plants, both of which are undesirable since the random nature of the crossing leads to lower yields (CFIA 1994). The commercial production of corn now utilises controlled cross-pollination of two inbred lines (using conventional techniques) to combine desired genetic traits and produce hybrid varieties known to be superior to open-pollinated varieties in terms of their agronomic characteristics.

This inbred-hybrid concept and resulting yield response is the basis of the modern corn seed industry and hybrid corn varieties are used in most developed countries for consistency of performance and production.

The host organism is a conventional corn hybrid line (LH244) resulting from a cross between the inbred lines LH197 and LH199 followed by a backcross to LH197. LH244 is a patented corn line assigned to Holden's Foundation Seeds LLC in 2001 (Armstrong 2001).

It is a medium season, yellow dent corn line that is adapted to the central regions of the U.S. corn-belt.

In terms of production, corn is the world's dominant cereal crop, ahead of wheat and rice and is grown in over 160 countries (FAOSTAT 2015). In 2013, worldwide production of corn was over I billion tonnes, with the United States and China being the major producers (~353 and 217 million tonnes, respectively) (FAOSTAT 2015). Corn is not a major crop in Australia or New Zealand and in 2012, production was approximately 506,000 and 201,00 tonnes respectively (FAOSTAT 2015).

Domestic production is supplemented by the import of corn grain and corn-based products, the latter of which are used, for example, in breakfast cereals, baking products, extruded confectionery and food coatings. In 2011, Australia and New Zealand imported, respectively, 856 and 5,800 tonnes of corn grain, 10,600 and 306 tonnes of frozen sweet corn and 8,427 and 900 tonnes of otherwise-processed sweet corn (FAOSTAT 2015). Corn product imports to Australia and New Zealand included 4,734 and 2,100 tonnes of corn flour and 1,520 and 13 tonnes of corn oil respectively (FAOSTAT 2015). Corn is a major source of crystalline fructose and high fructose corn syrup, both of which are processed from cornstarch. Approximately 3,000 tonnes of crystalline fructose, but negligible high fructose corn syrup, were imported into Australia in 2011 (Green Pool 2012); neither Australia nor New Zealand currently produce fructose (either crystalline or as high fructose corn syrup).

The majority of grain and forage derived from corn is used as animal feed, however corn also has a long history of safe use as food for human consumption. There are five main types of corn grown for food:

- Flour – *Zea mays* var. *amylacea*
- Flint – *Z. mays* var. *indurata*
- Dent – *Z. mays* var. indentata
- Sweet – *Z. mays* var. *saccharata* & *Z. mays* var. *rugosa*
- Pop – *Z. mays* var. *everta*

Dent corn is the most commonly grown for grain and silage and is the predominant type grown in the U.S. (OGTR 2008). MON87411 is a dent corn but could be crossed with other types.

Two main grain processing routes are followed for dent corn (White and Pollak 1995):

- Dry milling that gives rise to food by-products such as flour and hominy grits.

- Wet milling (CRA 2006), that involves steeping the grain, coarse and fine grinding, centrifugation and evaporating the steep, to yield food by-products such as starch (for cornstarch, corn syrup and individual sweeteners such as dextrose and fructose) and germ (for oil) – see Figure 1. Corn products are used widely in processed foods.

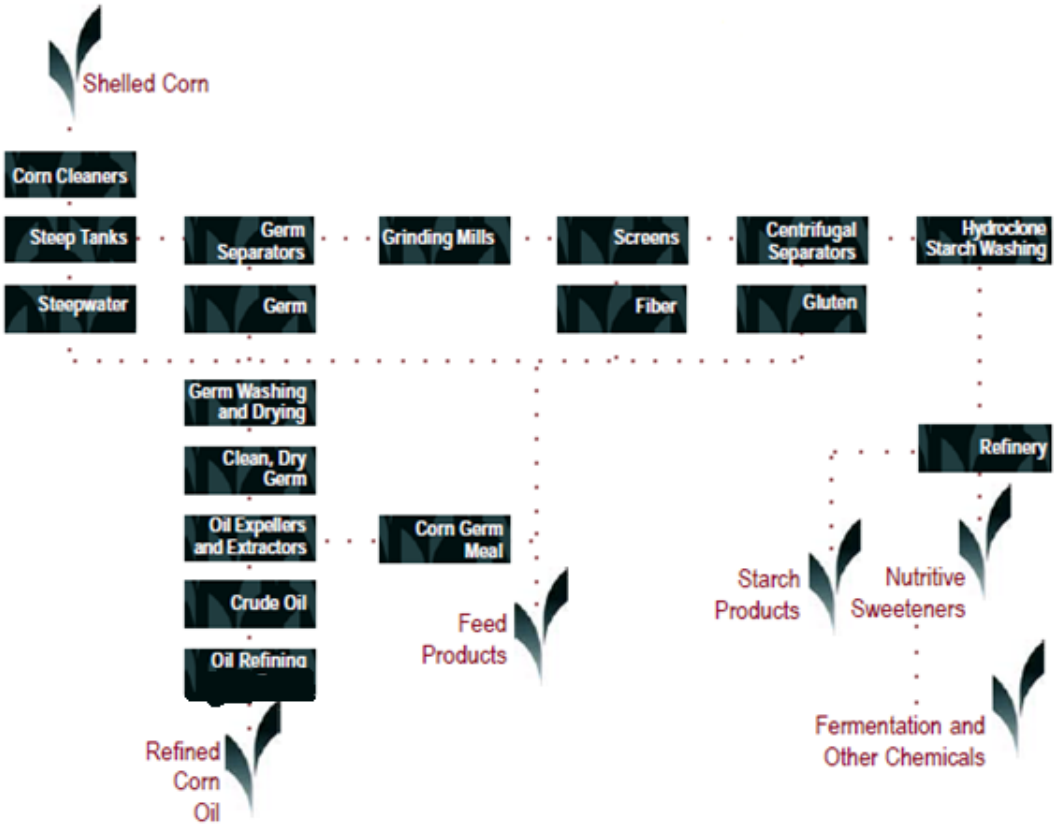


Figure 1: The corn wet milling process (diagram taken from CRA (2006))

2.2 Donor organisms

2.2.1 *Diabrotica virgifera virgifera*

The source of the *DvSnf7* fragment is the western corn rootworm (*Diabrotica virgifera virgifera*), a beetle (order Coleoptera, Family Chrysomelidae, Subfamily Galerucinae) that is native to North America but has now spread to Europe (Rabitsch 2006). This insect is a member of the corn rootworm complex that also includes the northern corn rootworm (*D. barberi*) and southern corn rootworm (*D. undecimpuntata howardi*). The insect larvae feed on corn roots causing both physiological damage to plants as a result of impaired water/nutrient absorption, and harvesting difficulties as a result of plant lodging. It is regarded as one of the most damaging insects to corn in the U.S. There are no reports of any direct effects of the insect on humans.

2.2.2 *Bacillus thuringiensis* subsp. *kumamotoensis*

Many different subspecies of *Bacillus thuringiensis (Bt)* have been isolated from dead or dying insects, mostly from the orders Coleoptera, Diptera and Lepidoptera, but many subspecies have also been found in the soil, aquatic environments and other habitats (WHO 1999). The source of the *cry3Bb1* gene used in MON87411 is the *Bt* subsp. *kumamotoensis* which is a spore-forming, gram-positive bacterium that is primarily associated with the soil and leaf surfaces.

Studies on mammals, particularly laboratory animals, demonstrate that *B. thuringiensis* is mostly non-pathogenic and non-toxic. *B. thuringiensis* has been demonstrated to be highly specific in its insecticidal activity and has demonstrated little, if any, direct toxicity to non-target insects (see NPTN 2000; OECD 2007 and references therein).

Infection in humans is unusual although there have been at least two clinical reports, one in the wounds of a soldier (Hernandez et al. 1998) and one in burn wounds (Damgaard et al. 1997), and in both cases impaired immunosuppression was implicated in the cause of the infection. *B. thuringiensis* has also been rarely associated with gastroenteritis (see eg Jackson et al. 1995) but generally, *B. thuringiensis* present in drinking water or food has not been reported to cause adverse effects on human health (WHO 1999; NPTN 2000; OECD 2007).

The effect of *B. thuringiensis* products on human health and the environment was the subject of a critical review by the WHO International Programme on Chemical Safety (WHO 1999). The review concluded that '*B. thuringiensis* products are unlikely to pose any hazard to humans or other vertebrates or the great majority of non-target invertebrates provided that they are free from non-*B. thuringiensis* microorganisms and biologically active products other than the insecticidal proteins'. Products containing *Bt* are approved for use on crops in Australia[1] and New Zealand[2] and in both countries there is an exemption from maximum residue limits (MRLs) when *Bt* is used as an insecticide[3].

2.2.3 *Agrobacterium* sp.

Agrobacterium sp. strain CP4 produces a naturally glyphosate-tolerant EPSPS enzyme and was therefore chosen as the gene donor for the glyphosate-tolerance trait. The bacterial isolate CP4 was identified in the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are known soil-borne plant pathogens but are not pathogenic to humans or other animals.

2.2.4 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of corn MON87411 (refer to Table 1). These non-coding sequences are used to drive, enhance, target or terminate expression of the novel genetic material. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from plant pathogens are not pathogenic in themselves and do not cause pathogenic symptoms in MON87411.

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.
- a characterisation of the dsRNA expressed in MON87411.

[1] https://portal.apvma.gov.au/pubcris
[2] http://www.biosecurity.govt.nz/pests-diseases/forests/white-spotted-tussock-moth/about-btk.htm
[3] New Zealand: http://www.foodsafety.govt.nz/elibrary/industry/nz-mrl-agricultural-compounds-food-standards-07-2014.pdf ; Australia: http://www.comlaw.gov.au/Details/F2014C00821

Studies submitted:

Carleton, S., C. Garnaat, K. Lawry, K. Skottke, Y. Yan and D. Kovalic. 2013. Amended Report for MSL0025048: Molecular Characterization of MON 87411. **MSL0025314.** Monsanto Company (unpublished)

Kang, H.T. and A. Silvanovich. 2013. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in MON 87411: Assessment of Putative Polypeptides. **MSL0024900**. Monsanto Company (unpublished)

Skottke, K., J. M. Ward and Q. Tian. 2013. Segregation of the T-DNA Insert in MON 87411 Across Three Generations. **MSL0024728.** Monsanto Company (unpublished)

3.1 Method used in the genetic modification

Immature embryos from line LH244 were aseptically removed from 10 – 13 day post-pollination ears and transformed, using a disarmed strain (ABI) of *Agrobacterium tumefaciens,* with the T-DNA from plasmid vector PV-ZMIR10871 (see Figure 2) following the method of Sidorov and Duncan (2009).

After co-culturing with the *Agrobacterium* carrying the vector, the embryos were placed on selection medium containing glyphosate, and carbenicillin, to inhibit the growth of untransformed plant cells and excess *Agrobacterium*, and to permit the development of callus tissue. Resulting callus was then placed in a medium that supported shoot regeneration and root development. Rooted plants (generation R_0) with normal phenotypic characteristics and tolerance to glyphosate were selected and transferred to soil for growth and further assessment. Following transformation, self-pollination, breeding, and segregation methods were used to produce MON 87411 (see Section 3.3).

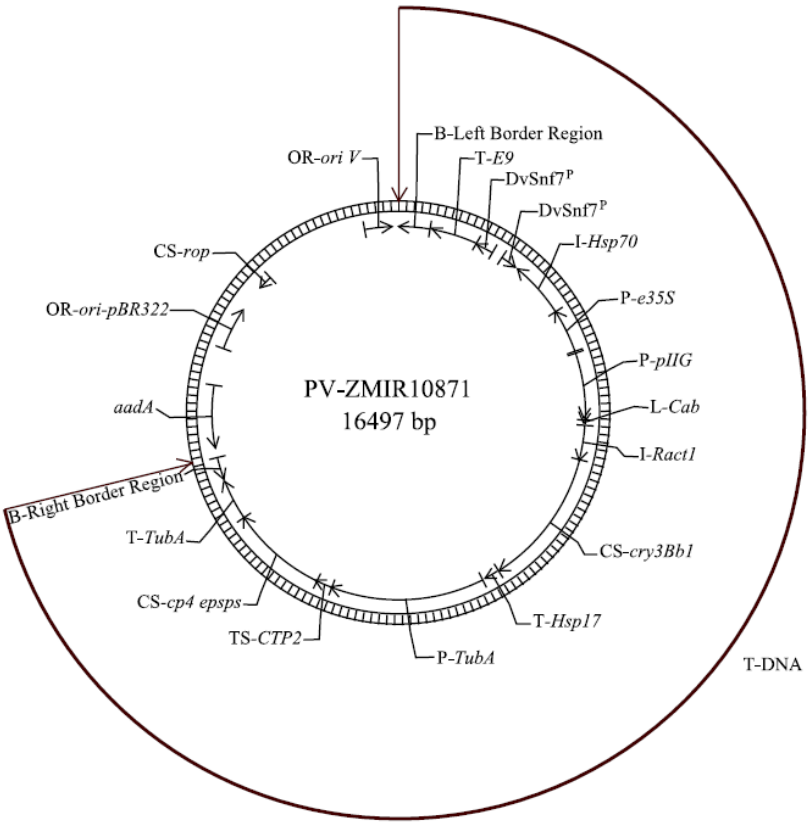


Figure 2: Genes and regulatory elements contained in plasmid PV-ZMIR10871

3.2 Function and regulation of introduced genetic material

Information on the genetic elements in the T-DNA present in PV-ZMIR10871 is summarised in Table 1. There are three cassettes comprising a total of 10,970 nt located between a 442 nt Left Border (LB) and a 331 nt Right Border (RB). The complete plasmid is 16,497 nt in size (i.e. the vector backbone comprises 4,753 nt).

Table 1: Description of the genetic elements contained in the T-DNA of PV-ZMIR10871

Genetic element	nt location on plasmid	Size (nt)	Source	Orient.	Description & Function	References
LEFT BORDER	1 - 442	442				
***DvSnf7* cassette**						
Intervening sequence	443 - 485	43				
E9	486 - 1118	633	*Pisum sativum* (pea)	Anti-clockwise	• 3' untranslated region of the *rbcS* gene family • Directs polyadenylation of the *DvSNf7* mRNA	Coruzzi *et al*. (1984)
Intervening sequence	1119 - 1147	29				
DvSnf7	1148 - 1387	240	*Diabrotica virgifera virgifera*	Anti-clockwise	• Partial coding sequence of the *Snf7* gene encoding the SNF7 subunit of the ESCRT-III complex that forms part of the suppression cassette	Baum *et al.* (2007a);Baum *et al*. (2007b);(Babst *et al*.(2002)
Intervening sequence	1388 - 1537	150				
DvSnf7	1538 - 1777	240	*Diabrotica virgifera virgifera*	Clockwise	• Partial coding sequence of the *Snf7* gene encoding the SNF7 subunit of the ESCRT-III complex that forms part of the suppression cassette	Baum *et al*. (2007a);Baum *et al*. (2007b);(Babst *et al*.(2002)
Intervening sequence	1778 - 1813	36				
Hsp70	1814 - 2617	804	*Zea mays* (corn)	Anti-clockwise	• Intron and flanking sequence of the *hsp70* gene encoding heat shock protein 70. • Involved in regulating gene expression	Rochester *et al.* (1986); Brown & Santino (1997)
e35S	2618 - 3238	621	Cauliflower Mosaic Virus (CaMV)	Anti-clockwise	• Promoter to direct transcription of *Snf7* • Also contains a duplicated enhancer region	Odell *et al*. (1985); Kay *et al.* (1987)
***cry3Bb1* cassette**						
Intervening sequence	3239 - 3264	26				
pIIG	3265 - 4213	949	*Zea mays* (corn)	Clockwise	• Promoter sequence from the physical impedance induced protein • Directs transcription of the *cry3Bb1* gene	Huang *et al.* (1998)
Intervening sequence	4214 - 4219	6				
Cab	4220 - 4280	61	*Triticum aestivum* (wheat)	Clockwise	• 5' untranslated region from the chlorophyll a/b binding (CAB) protein • Regulates gene expression	Lamppa *et al.* (1985)
Intervening sequence	4281 - 4296	16				
Ract1	4297 - 4776	480	*Oryza sativa* (rice)	Clockwise	• Intron and flanking untranslated region of the *act1* gene encoding rice Actin 1 protein • Involved in regulating gene expression	McElroy *et al*. (1990)
Intervening sequence	4777 - 4785	9				

Genetic element	nt location on plasmid	Size (nt)	Source	Orient.	Description & Function	References
cry3Bb1	4786 - 6747	1,962	*Bacillus thuringiensis subspecies kumamotoensis*	Clockwise	• Codon-optimised coding sequence for the Cry3Bb1 protein	English *et al*. (2000)
Intervening sequence	6748 - 6766	19				
Hsp17	6767 - 6976	210	*Triticum aestivum* (wheat)	Clockwise	• 3' untranslated region from the gene encoding heat shock protein 17. • Directs polyadenylation of the *cry3Bb1* mRNA	McElwain & Spiker (1989)
***cp4 epsps* cassette**						
Intervening sequence	6977 - 7024	48				
TubA	7025 - 9205	2,181	*Oryza sativa* (rice)	Clockwise	• 5' untranslated region leader and intron sequences from the *OsTubeA* gene encoding α-tubulin • Directs transcription of the *cp4 epsps* gene	Jeon et al. (2000)
Intervening sequence	9206 - 9209	4			•	
CTPT2	9210 - 9437	228	*Arabidopsis thaliana*	Clockwise	• Targeting sequence of the *ShkG* gene encoding the EPSPS transit peptide • Directs transport of the CP4 EPSPS protein to the chloroplast	Herrmann (1995); Klee *et al.* (1987)
cp4 epsps	9438 - 10805	1,368	*Agrobacterium* sp. strain CP4	clockwise	• Codon optimised coding sequence of the *aroA* gene encoding the CP4 EPSPS protein	Padgette et al (1996); Barry et al (2001)
Intervening sequence	10806 - 10812	7				
TubA	10813 - 11394	582	*Oryza sativa* (rice)	clockwise	• 3' untranslated region sequence from the *OsTubeA* gene encoding α-tubulin • Directs polyadenylation of the *cp4 epsps* gene	Jeon et al. (2000)
Intervening sequence	11395 - 11412	18				
RIGHT BORDER	11413 - 11743	331				

3.2.1 *DvSnf7* expression cassette

The *DvSnf7* sequence contained in the expression cassette is a partial coding sequence of the *DvSnf7* gene from *Diabrotica virgifera virgifera* (western corn rootworm). The cassette contains two 240 nt *DvSnf7* fragments in an inverted orientation, driven by the constitutive *e35S* promoter (with a duplicated enhancer region) from Cauliflower mosaic virus. An intron (*hsp70*) from *Zea mays* (corn) enhances expression. Between the two inverted fragments is a 150 nt intervening sequence that facilitates the formation of a hairpin loop following transcription, leading to the formation of double-stranded *DvSnf7* RNA. A sequence from the E9 3' untranslated region of the *ribulose bisphosphate carboxylase* gene from *Pisum sativum* (pea) terminates transcription.

3.2.2 cry3Bb1 expression cassette

The *cry3Bb1* gene was optimised for expression in plant cells (English et al. 2000). The gene is under the control of the constitutive *pIIG* promoter derived from *Z. mays*. A leader sequence (*Cab*) from *Triticum aestivum* (wheat) and an intron (*Ract1*) from *Oryza sativa* (rice) both help to enhance gene expression. A sequence from the 3' untranslated region of the *Hsp17* gene from *T. aestivum* terminates transcription.

3.2.3 *cp4 epsps* expression cassette

The *cp4 epsps* gene was initially isolated and cloned from the bacterium *Agrobacterium* sp. strain CP4. The gene has been optimised for expression in plants (Padgette et al. 1996; Barry et al. 2001). Expression of the gene confers tolerance to the herbicide glyphosate.

The *cp4 epsps* coding region is 1,368 nt in length and is driven by the constitutive *TubA* promoter from *O. sativa*. The addition of the leader and intron from *TubA* enhances expression. A transit peptide (CTPT), derived from elements from the *ShkG* gene from *Arabidopsis thaliana,* targets the CP4 EPSPS protein to the chloroplasts. The CTPT is typically cleaved on uptake of the mature protein into the chloroplast, and is subsequently rapidly degraded. A sequence from the *TubA* 3' non-translated region functions to terminate transcription of the *cp4 epsps* gene and direct polyadenylation of the mRNA.

3.3 Breeding of corn line MON87411

The breeding pedigree for the various generations is given in Figure 3.

R_0 plants (numbering in the hundreds) generated through the transformation process described in Section 3.1 were self-pollinated over six generations in order to produce homozygous lines. At each generation, the progeny were evaluated for desirable molecular and phenotypic characteristics. MON87411 was selected as the lead event, based on its insert integrity, glyphosate tolerance, efficacy against CRW larval damage and superior phenotypic characteristics.

Seed from the R_4 and R_5 generations was used in trait integration and further commercial development through crosses with conventional inbred lines (HCL645 and LH287).

Based on seed availability and appropriate fit for various studies, non-GM hybrid maize lines MPA640B (LH244 x LH287) and NL6169 (LH244 x HCL645) were used as controls in addition to the non-GM parental hybrid line LH244 (see Table 2).

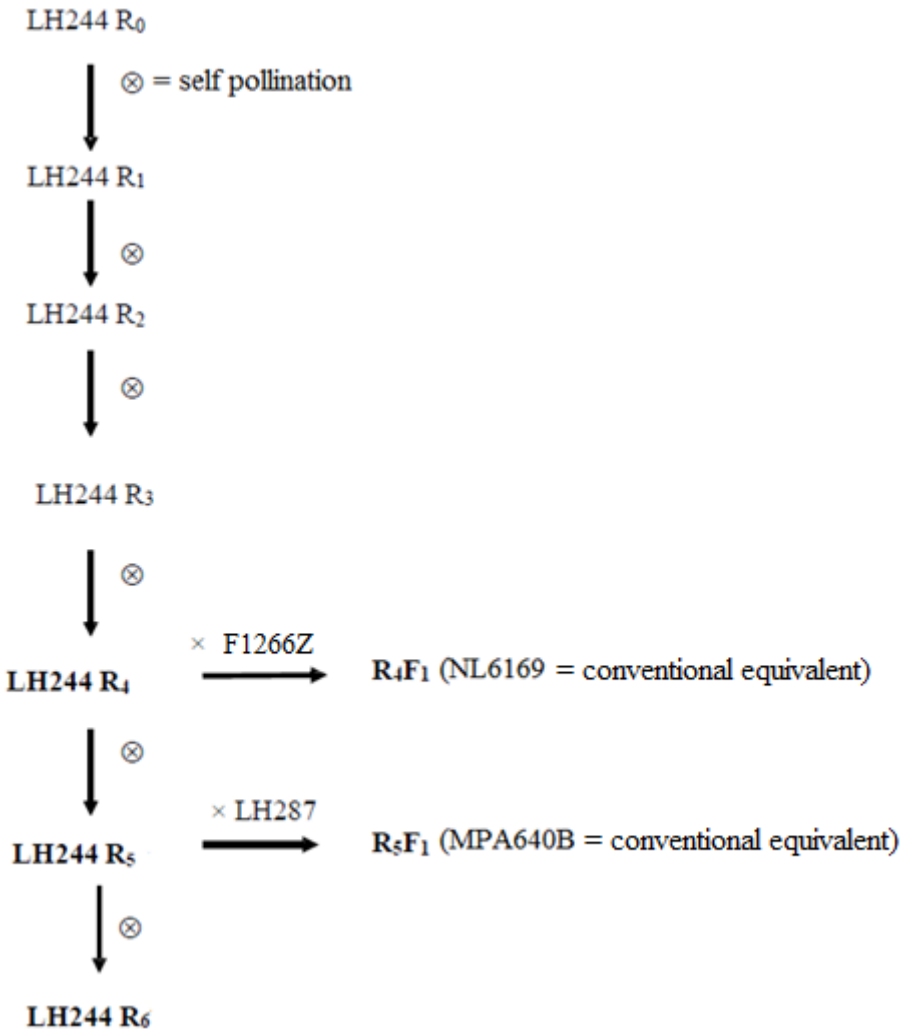


Figure 3: Breeding diagram for MON87411

Table 2 indicates the generations and controls that were used in the various studies characterising MON87411.

Table 2: MON87411 generations used for various analyses

Analysis	MON87411 Generation used	Control(s) used
Molecular characterisation	R_4	LH244
Mendelian inheritance	BC_2F_1, BC_2F_2, BC_3F_1 (see Figure 6)	N/A
Genetic stability	R_4, R_5, R_6, R_4F_1, R_5F_1	LH244; MPA640B; NL6169
Protein expression levels in plant parts	R_4F_1	LH244
Protein characterisation	R_4F_1	*E. coli*
RNA expression in the plant	R_4F_1	LH244
Compositional analyses	R_4F_1	NL6169

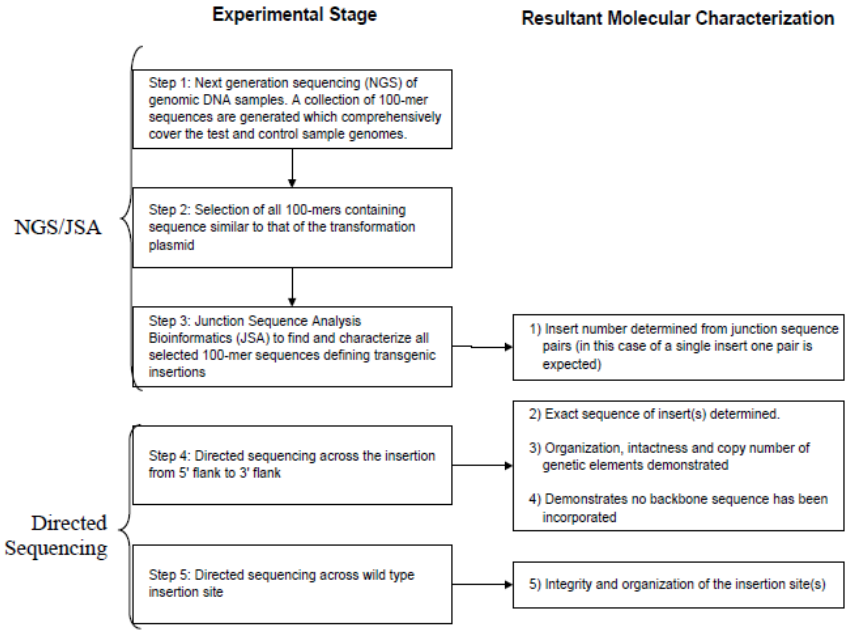
3.4 Characterisation of the genetic modification in the plant

A range of analyses were undertaken to characterise the genetic modification in corn line MON87411. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure.

The molecular characterisation of MON87411 incorporated a new approach (Kovalic et al. 2012; DuBose et al. 2013) that applies Next Generation Sequencing (NGS) and Junction Sequence Analysis (JSA) together with bioinformatics to determine the number of inserts. In the past, this has been determined by Southern blot analysis. The organisation and sequence of the insert and adjacent flanking DNA, and the sequencing of the insertion site were all determined by methods employing directed sequencing.

The rationale for junction sequence analysis is that, since junctions are characteristic of DNA insertion, it follows that each insertion will produce two (i.e. 5' and 3') unique junction sequences. By evaluating the number of unique junctions detected, the number of insertion sites can be determined.

The Applicant supplied the flow-diagram shown in Figure 4 to illustrate this new approach to molecular characterisation.



Genomic DNA from MON 87411 and the conventional control was sequenced using NGS technology that produces a set of short, randomly distributed sequence reads (each approximately 100 bp long) that comprehensively cover the genomes (Step 1). Utilizing these genomic sequences, bioinformatics search tools were used to select all sequence reads (100-mers) that were significantly similar to the transformation plasmid (Step 2) and Junction Sequence Analysis (JSA) bioinformatics was used to determine the insert number (Step 3). Overlapping PCR products are produced which span any insert(s) and their wild type loci (Step 4 and Step 5, respectively). These PCR products are sequenced to provide a detailed characterization of the insertion site(s).

Figure 4: Steps in the molecular characterisation of MON87411

3.4.1 Insert number

Total genomic DNA from grain of verified MON87411 (generation R_4) and the untransformed parent (LH244) was sequenced using Illumina®[4] NGS technology. Reference DNA was also used from the plasmid vector PV-ZMIR10871. As a positive control, plasmid DNA was spiked into LH244 DNA at a single copy genome equivalent ratio and 1/10 copy genome equivalent ratio. It was noted from the subsequent positive control results, that any portion of the plasmid could be detected at both single copy and 1/10 copy; this indicated there was adequate sensitivity to be able to observe any inserted fragment.

The DNA was sheared into approximately 325 bp fragments, processed for deep sequencing (end-repaired, A-tailed and ligated to adapters), enriched through ten cycles of polymerase chain reaction (PCR) and then sequenced using Illumina HiSeq® technology that produces short-sequence reads approximately 100 bp long. To confirm sufficient sequence coverage in the samples, the 100-mer sequence reads from all samples were analysed to determine the effective depth of coverage (i.e. the average number of times any base of the genome is expected to be independently sequenced) by mapping all reads to a known single-copy endogenous corn pyruvate decarboxylase gene (*pdc3*). The analysis showed that *pdc3* was covered by the 100-mers at >107x for each sample, a coverage that is considered to be comprehensive (Kovalic et al. 2012).

An *in silico* analysis using the BLAST[5] algorithm then followed, in which only those 100-mer reads containing sequence similarity to the plasmid PV-ZMIR10871 were selected i.e. this analysis found all 100-mer reads that were either fully matched to the insert plasmid sequences or contained both plasmid sequences and junction sequences. The analysis collected all sequencing reads with an e-Value[6] of less than 1e-5 and at least 30 bases match of greater than 96.7% identity to the transformation plasmid (Kovalic et al. 2012). Using Bowtie[7] short sequence alignment software, non-duplicated reads of $\geq$30 nt were collected. Following *in silico* adapter removal (Novoalign software[8]) and *in silico* removal of low quality read ends (Phred score[9] < 12) the remaining reads were then aligned to the whole plasmid PV-ZMIR10871 sequence in order to find junction region sequences (Figure 5). Reads were also aligned against the control genome in order to remove those reads sourced from endogenous homologues.

Figure 5 shows a map of the junction sequences (illustrated as stacked bars) that were detected. Each detected junction sequence read is shown trimmed to include only 30 nt of plasmid sequence. Only two unique junction sequence classes, both containing portions of T-DNA and flanking sequence were detected. This indicates that MON87411 contains a single DNA insert. No junction sequences were found in the DNA from LH244.

[4] http://www.illumina.com/technology/next-generation-sequencing/sequencing-technology.ilmn
[5] BLAST is the acronym for Basic Local Alignment Search Tool (Altschul et al. 1990), a computer algorithm that can rapidly align and compare a query DNA sequence with other DNA sequences..
[6] Comparisons between highly homologous proteins yield *E*-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger *E*-value indicates a lower degree of similarity.
[7] http://bowtie-bio.sourceforge.net/index.shtml
[8] http://www.novocraft.com/main/index.php
[9] Phred is a base calling programme for DNA sequences. Phred quality scores have become widely accepted to characterise the quality of sequences (http://www.phrap.com/phred/) .

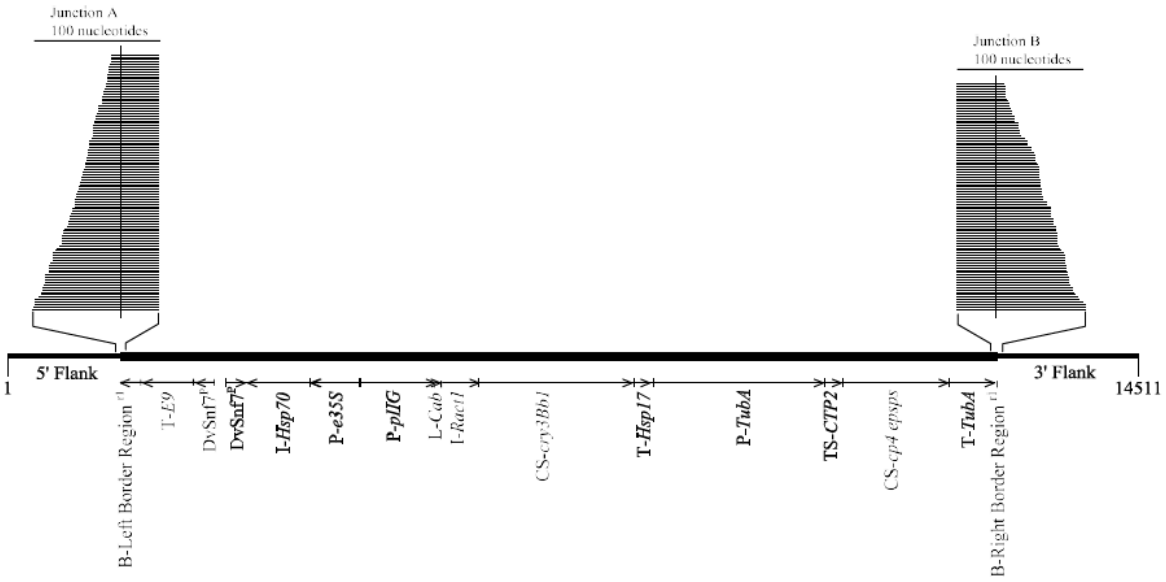


Figure 5: Schematic representation of the junction sequences detected in MON87411

3.4.2 Insert organisation and sequence

PCR primers were designed to amplify eight overlapping regions of MON87411 genomic DNA incorporating the insert and flanking regions [no products were obtained for DNA from LH244]. The products were used to determine the nucleotide sequence of the insert and flanking regions using BigDye® Terminator chemistry[10]. A consensus sequence was generated by compiling sequences from multiple sequencing reactions performed on the overlapping PCR products. The consensus sequence was then aligned to the PV-ZMIR10871 sequence to determine the integrity and organisation of the insert and flanking regions.

The results showed that the insert is 11,248 nt in length and comprises the identical 10,970 nt sequence found within the T-DNA of plasmid PV-ZMIR10871 together with terminally truncated Right and Left Border regions (RB missing the last 316 nt and LB missing the first 179 nt – see Table 1 for details of full sequence length). This analysis also showed that no plasmid backbone sequences are present in MON87411, and confirmed the conclusion from the NGS/JSA analysis that a single copy of the T-DNA has been inserted.

In addition to the insert, 1,464 nt flanking the 5' end of the insert and 1,803 nt flanking the 3' end of the insert were sequenced.

3.4.3 The insertion site

In order to identify any changes to the genomic DNA as a result of the insertion event, two primers (one specific to the 5' flanking sequence of MON87411 and one specific to the 3' sequence) were used for PCR of genomic DNA isolated from the untransformed parent (LH244). The product (approximately 3,500 nt) was then sequenced and the sequence was compared with the sequences obtained for the 5' and 3' flanking regions of MON87411. The results showed that a 118 bp deletion (bases 1453 – 1570 of LH244) had occurred during transformation. Deletions such as this are not uncommon and most likely result from double-stranded break repair in the plant during the *Agrobacterium*-mediated transformation process (Salomon and Puchta 1998).

[10] http://www.appliedbiosystems.com.au/

3.4.4 Open reading frame (ORF) analysis

Sequences spanning the 5' and 3' junctions of the MON87411 insert were translated using DNAStar software[11] from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames. A total of eight ORFs (five in the 5' junction and three in the 3' junction) were identified that encode putative polypeptides ranging in size from 15 – 87 amino acids. No analysis was done to determine whether any potential regulatory elements were associated with the polypeptides.

The putative polypeptides encoded by the eight identified ORFs were then analysed using a bioinformatic strategy to determine similarity to known protein toxins or allergens (refer to Section 4.1.5).

3.5 Stability of the genetic changes in corn line MON87411

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 event) over successive generations. It is best assessed by molecular techniques, such as Southern blot analysis or NGS/JSA. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations. It is often quantified by a trait inheritance analysis to determine Mendelian heritability via assay techniques (chemical, molecular, visual).

3.5.1 Genetic stability

The genetic stability of event MON87411 was evaluated by NGS/JSA (as described in Section 3.4.1) in verified genomic DNA isolated from the grain of plants of five generations (refer to Figure 3) as given in Table 3. Control genomic DNA was isolated from the non-GM parental line and two other conventional lines as indicated in Table 3.

Table 3: Source of genomic DNA used for genetic stability analysis

DNA source	Generation
MON87411	R_4
	R_5
	R_6
	R_4F_1
	R_5F_1
LH244	
NL6169	
MPA640B	

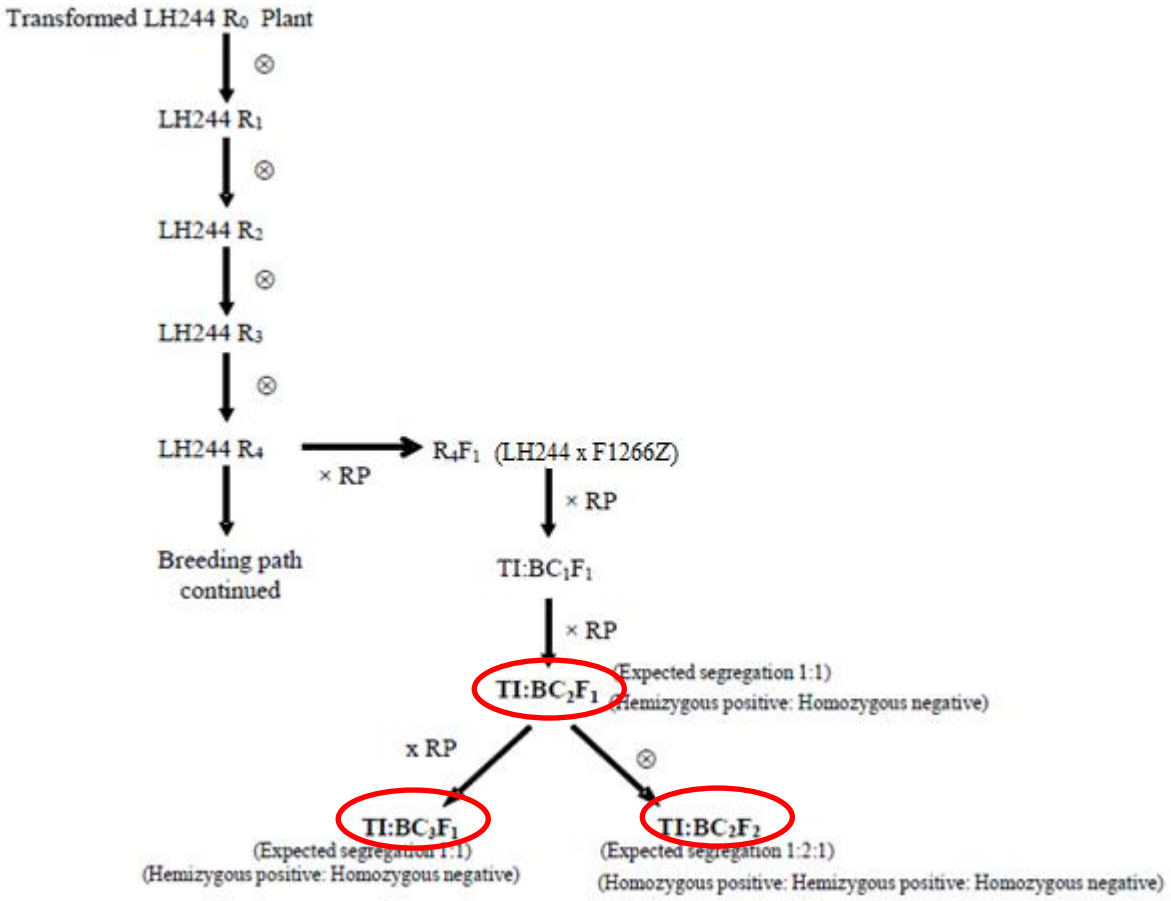
No junction sequences were detected in DNA obtained from the control lines (LH244, NL6169 and MPA640B). Analysis of the MON87411 DNA from all generations showed the presence of the same two junction sequences identified in Section 3.4.1. No other junction sequences were present. The consistency of this junction sequence data across all generations tested, demonstrates that the single insert is stably maintained in MON87411.

3.5.2 Phenotypic stability

Since it was demonstrated that the insert resides at a single locus within the MON87411 genome, the expectation would be that the genetic material within it would be inherited according to Mendelian principles.

[11] http://www.dnastar.com/

Chi-square (X^2) analysis was undertaken over several generations to confirm the segregation and stability of the complete T-DNA sequence within the insert. The breeding path followed for this analysis was different from that represented in Figure 3 and is shown in Figure 6. Basically, at each stage, a Real-Time TaqMan® PCR assay was used to select plants containing the T-DNA. Ultimately, the inheritance of the T-DNA was assessed in the BC_2F_1, BC_2F_2 and BC_3F_1 generations (in which the GM parent had been hemizygous for the MON87411 T-DNA). At the BC_2F_1 and BC_3F_1 generations, the MON 87411 T-DNA was predicted to segregate at a 1:1 ratio (hemizygous positive: homozygous negative) according to Mendelian inheritance principles. At the BC_2F_2 generation, the MON 87411 T-DNA was predicted to segregate at a 1:2:1 ratio (homozygous positive: hemizygous positive: homozygous negative) according to Mendelian inheritance principles.



RP: Recurring parent = F1266Z (i.e. does not contain *DvSnf7*, *cp4 epsps* or *cry3Bb1*)
⊗=Self-Pollinated
TI = Trait integration (replacement of genetic background of MON87411 by recurrent background except inserted gene)

Figure 6: Breeding path for generating segregation data for MON87411

The results (Table 4) indicated there were no significant differences between the observed and expected segregation ratios in any of the generations. This supported the conclusion that the T-DNA resides at a single locus and showed that the T-DNA is inherited according to Mendelian principles.

Table 4: Segregation of the MON87411 T-DNA sequences over three generations

Generation	Total plants	Ratio[1]		X^2	Probability (P)[2]
		Observed	Expected		
BC_2F_1	351	1:1.04	1:1	0.14	0.709
BC_3F_1	223	1:1.14	1:1	1.01	0.315
BC_2F_2	623	1:2.06:1.03	1:2:1	0.12	0.942

[1] For BC2F1 and BC3F1, ratio is hemizygous positive:homozygous negative for each of observed and expected
For BC2F2, ratio is homozygous positive:hemizygous:homozygous negative for each of observed and expected
[2] Statistical significance is when P≤0.05

3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in corn line MON87411. The insert sequence analysis (Section 3.4.2) showed no plasmid backbone has been integrated into the MON87411 genome during transformation, i.e. the *aadA* gene, which was used as a bacterial selectable marker gene, is not present in corn MON87411.

3.7 *DvSnf7* RNA expression in MON87411

The full length transcript produced in MON87411 would be expected to be around 1,000 bp in size.

Study submitted

Carleton, S., Y. Wang, J. Ward and Q. Tian. 2013. Northern Blot Analysis of DvSnf7 RNA Expression in MON 87411. **RAR-2013-0213**. Monsanto Company (unpublished).

Northern blot analyses were undertaken in order to confirm whether the transcript produced in MON87411 by the *DvSnf7* expression cassette has the expected size and whether the expected 240 bp dsDvSnf7 RNA is also present.

Total RNA was isolated from V6 – V8 stage leaf tissue of verified MON87411 (generation R_4F_1) and the parent control LH244. The RNA was either left undigested or was digested with a commercial RNase I_f[12]. The RNA was then electrophoresed on an agarose/formaldehyde gel and transferred to a nylon membrane for blotting. DNA from plasmid PV-ZMIR10871 was used to generate template for a 240 nt DvSnf7 radiolabeled (^{32}P) probe.

No hybridisation bands were detected for either undigested or digested RNA from LH244. Undigested RNA from MON87411 showed a single band at approximately 1,000 nt confirming that MON87411 produces the full-length DvSnf7 transcript. RNase I_f digested RNA from MON87411 showed a single hybridisation band at 240 bp, indicating that, following transcription, transcribedDvSnf7 RNA exists in the plant as a double-stranded form that is of the expected size. It was not possible to determine from these results if intermediate low abundant forms of DvSnf7 RNA in single-stranded form exist.

[12] Ribonuclease If (RNase I_f) is an RNA endonuclease which will cleave at all RNA dinucleotide bonds leaving a 5´ hydroxyl and 2´, 3´ cyclic monophosphate (http://www.neb.com/~/media/Catalog/All-Products/A2A413C40FD3447B9EFA2F148FB9FBBB/Datacards%20or%20Manuals/M0243Datasheet-Lot0111205.pdf). It will digest only single-stranded RNA and leave double-stranded RNA intact.

In addition to the northern analysis, cDNA sequencing reported in a published study (Armstrong et al. 2013) has indicated that the primary RNA transcript accumulating in MON87411 is a 968 nt fragment comprising the two 240 nt inverted repeats and adjacent 5'- and 3' sequences. The full-length (approximately 1.2 kb) mRNA consists of the 968 nt primary transcript that has been post-transcriptionally modified to include a poly-A tail and 5' cap. The exact number of adenosine nucleotides in the pol-A tail cannot be accurately determined but has been estimated to be about 200 (Armstrong et al 2013).

3.8 Conclusion

Corn line MON87411 contains three expression cassettes: the *DvSnf7* cassette; the *cry3Bb1* cassette; and the *cp4 epsps* cassette.

Comprehensive molecular analyses of MON87411 indicate there is a single insertion site comprising a single, complete copy of each of the three expression cassettes. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and directed sequencing analysis shows no plasmid backbone has been incorporated into the transgenic locus.

The results from both the insert sequence analysis (Section 3.4.2) and the phenotypic stability analysis (Section 3.5.2) are consistent with the results from NGS/JSA which was used to determine the number of inserts.

Northern blot analysis of MON87411 RNA shows that a full-length single-stranded DvSnf7 transcript (comprising a 968 nt primary transcript, poly-A tail and 5' cap) is produced upon expression of the *DvSnf7* cassette and that this then leads to the formation of a 240 bp dsDvSnf7 RNA, which is the expected size.

4 Characterisation and safety assessment of new substances

4.1 Newly expressed proteins

In considering the safety of novel proteins it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g., because they are allergens or anti-nutrients (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-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

Two types of novel proteins were considered:

- Those that were expected to be directly produced as a result of the translation of the introduced genes. A number of different analyses were done to characterise these proteins and determine *in planta* expression.

- Those that may be potentially translated as a result of the creation of ORFs during the transformation process (see Section 3.4.4).

4.1.1 The Cry3Bb1 and CP4 EPSPS proteins

Cry proteins produced by *Bacillus thuringiensis* (*Bt*) are classified by their primary amino acid sequence and more than 500 different *cry* gene sequences have been classified into 73 groups (Cry1–Cry73)[13]. The largest family is the 3D-Cry group and contains proteins subdivided into further groups based on their specificity for target insects; Cry 3 proteins (such as Cry3Bb1) act specifically on Coleopterans while Cry1A proteins, for example, act specifically on Lepidopterans (Höfte and Whiteley 1989).

The primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells, which subsequently leads to ion leakage and cell lysis. The crystal inclusions ingested by susceptible larvae dissolve in the alkaline environment of the gut, and the solubilised inactive protoxins are cleaved by midgut proteases yielding 60-70 kDa protease resistant core toxins (Bravo et al. 2007). Toxin activation involves the proteolytic removal of an N-terminal peptide. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells (Hofmann et al. 1988; Aronson and Shai 2001) before inserting into the membrane. Toxin insertion leads to formation of lytic pores in microvilli apical membranes (de Maagd et al. 2001; Aronson and Shai 2001) and eventually to cell lysis and disruption of the gut epithelium. The septicaemia that inevitably follows may be mediated by an influx of enteric bacteria into the haemocoel (Broderick et al. 2006).

EPSPS proteins occur ubiquitously in plants and microorganisms and have been extensively studied over a period of more than thirty years. EPSPS catalyses a step in the shikimate pathway that is responsible for the biosynthesis of the aromatic amino acids phenylalanine, tryptophan and tyrosine. Inhibition of this pathway is lethal. In plants, the EPSPS enzyme is inhibited by glyphosate (Steinrucken and Amrhein 1980), but bacterial EPSPSs, such as the CP4 EPSPS, have a reduced affinity for glyphosate (Padgette et al. 1996; Barry et al. 2001) thereby allowing the continued action of the enzyme in the presence of glyphosate. A single residue in the active site (Ala-100) renders the CP4 EPSP synthase insensitive to glyphosate, whereas a highly conserved Gly residue is found at this position in known natural plant and bacterial enzymes (Funke et al. 2006).The CP4 EPSPS protein present in corn MON87411 is functionally the same as the *Agrobacterium* enzyme. The LH244 corn line used as the parent for the genetic modification described in this application, also contains the native EPSPS protein (that is inhibited by glyphosate).

4.1.2 Cry3Bb1 and CP4 EPSPS expression in MON87411 tissues

Study submitted:

Beyene, A. 2013. Assessment of Cry3Bb1 and CP4 EPSPS Protein Levels in Corn Tissues Collected from MON 87411 Produced in Argentina Field Trials during 2011-2012. **MSL0024586**. Monsanto Company.

Plants of MON87411 (generation R_4F_1) and LH244 were grown from verified seed lots at five field sites in Argentina[14] during the 2011-2012 growing season. Four of these plantings overlapped with those used for the compositional analyses described in Section 5. The identity of any subsequent harvested grain from each site was also confirmed using event-specific polymerase chain reaction (PCR). There were four replicated plots at each site planted in a randomised complete-block design. The MON87411 plots were sprayed, at the 2-4 leaf stage with glyphosate herbicide.

[13] http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html
[14] 2 x sites at Pergamino, Buenos Aires; Hunter, Buenos Aires; Sarasa, Buenos Aires; Salto, Buenos Aires.

Samples were taken at various stages of growth (Table 5) and the levels of Cry3Bb1 and CP4 EPSPS proteins were determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA). Detection of the Cry3Bb1 protein utilised a polyclonal biotin-coupled goat anti-Cry3Bb1 capture antibody and a NeutrAvidin (Thermo Fisher Scientific) detection reagent conjugated to horseradish peroxidase. CP4 EPSPS was detected using a monoclonal mouse anti-CP4 EPSPS capture antibody and a commercial detection reagent (Sigma-Aldrich) comprising goat-anti CP4 EPSPS conjugated to horseradish peroxidase. Plates were analysed on a commercial microplate spectrophotometer (SPECTRAmax Plus 384, Molecular Devices). Quantification of each protein was accomplished by interpolation on a protein standard curve.

The results, averaged over all sites, are given in Table 5.

Table 5: Cry3Bb1 and CP4 EPSPS protein content in MON87411 corn parts at different growth stages (averaged across 5 sites)

Tissue/Growth stage[1]	Days after planting	Cry3Bb1 µg/g dry weight		CP4 EPSPS µg/g dry weight	
		Mean (SD)	Range	Mean (SD)	Range
Leaf /V3 – V4	21 - 22	270 (65)	160 - 390	42 (59)	33 - 55
Leaf /V6 – V8	35 - 44	210 (40)	120 - 270	36 (3.1)	29 – 39
Leaf /V10 – V13	50 - 55	170 (35)	92 - 220	32 (3.8)	27 – 42
Leaf /V14 – R1	59 -78	220 (63)	130 - 340	31 (3.5)	24 – 37
Root /V3 – V4	21 - 22	180 (43)	130 - 280	48 (6.6)	38 – 63
Root /V6 – V8	35 - 44	120 (24)	67 - 170	37 (7)	23 – 48
Root /V10 – V13	50 - 55	84 (21)	54 - 130	31 (4.7)	24 – 37
Root /V14 – R1	59 - 78	75 (19)	43 - 120	30 (4.8)	20 – 38
Whole plant /V3 – V4	21 - 22	340 (49)	250 - 460	63 (6.7)	54 – 76
Whole Plant /V6 – V8	35 - 44	190 (30)	130 - 270	36 (5.8)	21 – 46
Whole Plant /V10 – V13	50 - 55	140 (39)	59 - 210	33 (6.2)	21 – 45
Whole Plant /V14 – R1	59 - 78	120 (28)	71 - 170	25 (5)	17 – 32
Stover /R6[2]	136 - 155	21 (13)	4.7 - 44	2.2 (1.2)	0.59 – 4.9
Senescent Root /R6	136 - 155	19 (13)	3 - 5	5.4 (2.9)	1.8 – 11
Forage Root /R5	101 – 111	36 (16)	13 – 66	10 (3.7)	5.1 – 19
Forage /R5[2]	101 – 111	39 (17)	18 – 75	8 (2.3)	5.2 – 13
Grain /R6	139 - 154	4 (0.56)	3.1 – 5.1	1.9 (0.31)	1.6 – 3.1
Pollen /VT – R1	65 - 80	36 (4)	30 - 42	19 (2.8)	16 – 24
Silk /R1	65 - 81	160 (37)	89 - 220	40 (5)	32 - 49

[1]For information on corn growth stages see e.g. Ransom & Endres (2014)
[2] Stover is the dried stalks and leaves of a field crop used as animal fodder after the grain has been harvested
[3]Forage is the above ground plant parts used for animal feed.

For both Cry3Bb1 and CP4 EPSPS, mean levels were lowest in the grain (4.0 and 1.9 µg/g dry weight, respectively). The highest levels of both proteins were in the whole plant samples at the V3 – V4 stages (340 and 63 µg/g dry weight, respectively) as would be expected from the high levels in both the leaves and roots at this stage. Values for both proteins obtained for tissue from LH244 were below the limit of detection (LOD) or limit of quantitation (LOQ) in all samples.

4.1.3 Characterisation of the Cry3Bb1 and CP4 EPSPS proteins

The wild type, full-length Cry3Bb1 protein from *B. thuringiensis* comprises 652 amino acids and has a molecular weight of approximately 74.4 kDa. (Donovan et al. 1992). The Applicant states the amino acid sequence of the Cry3Bb1 protein in MON87411 is identical to that of the Cry3Bb1 protein assessed by FSANZ in MON88017 (FSANZ 2006a).

In that line, the Cry3Bb1 coded for was a variant that consisted of 653 amino acid residues (mw ≈ 77 kDa) due to an additional residue (alanine) at position 2 in the protein, which resulted from the need to create a DNA restriction enzyme site for laboratory manipulations. In addition, there are five other specified amino acid changes in the protein expressed in the two corn lines compared to the wild-type *Bt* protein. The changes amount to 99.1% amino acid identity of the Cry3Bb1 variant with the wild-type Cry3Bb1 protein. The amino acid sequence of the Cry3Bb1 variants present in MON87411 and MON88017 and a previously assessed line, MON 863 (FSANZ 2003), share 99.8% identity; and differ by only one of 653 amino acid residues. The full-length Cry3Bb1 protein is partially processed in the plant (FSANZ 2006a) to produce proteins with apparent molecular weights of ~66 and ~55 kDa. The ~55 kDa protein is thought to correspond to the tryptic core of the protein described in the literature (Schnepf et al. 1998).

The CP4 EPSPS protein found in *Agrobacterium* sp. strain CP4 contains 455 amino acids (Padgette et al. 1996). It is functionally the same as the EPSPS protein naturally present in plants and microorganisms (Franz et al. 1997), except for the modification to glyphosate susceptibility, and has approximately 30% amino acid similarity with plant EPSPS enzymes (Harrison et al. 1996). It is expected to be a 47.6 kDa protein. In MON87411, the pre-protein consists of 531 amino acids including the CTP2 transit peptide of 76 amino acids, which is cleaved on uptake into the plant chloroplasts.

It is necessary to confirm that the proteins expressed in MON87411 have the expected biochemical characteristics. Accordingly, the Applicant used a number of analytical techniques to characterise the Cry3Bb1 and CP4 EPSPS proteins and compare them with previously characterised microbially-derived proteins

The techniques used were:

- Sodium dodecyl polyacrylamide gel electrophoresis (SDS - PAGE)
- Western blot analysis
- N-terminal sequencing
- Peptide mass mapping
- Glycosylation analysis
- Enzyme bioactivity assay

Studies submitted:

Hernan, R., R. Heeren and G. Mueller. 2013. Characterization of the Cry3Bb1 Protein Purified from the Maize Grain of MON 87411 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*- Produced Cry 3Bb1 Proteins. **MSL0024872**. Monsanto Company (unpublished).

Lee, T.C. and S. B. Storrs. 2013. Characterization of the CP4 EPSPS Protein Purified from the Maize Grain of MON 87411 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*- Produced CP4 EPSPS Proteins. **MSL0024834**. Monsanto Company (unpublished).

Plant-produced Cry3Bb1 and CP4 EPSPS proteins were immuno-purified from grain of MON87411 (generation R_4F_1). Several batches of each protein were prepared in order to generate sufficient amounts of pooled protein. Microbially-derived Cry3Bb1 and CP4 EPSPS had previously been produced and characterised from *Escherichia coli*.

4.1.3.1 Molecular weight and immunoreactivity of Cry3Bb1 and CP4 EPSPS

The molecular weights of plant- and microbially-derived proteins were estimated from SDS-PAGE. Following electrophoresis, gels were stained with Brilliant Blue G-Colloidal stain and analysed by densitometry.

Cry3Bb1

For protein obtained from MON87411, a number of bands on the stained SDS-PAGE gel were visible, the most prominent of which had apparent molecular weights of approximately 77 kDa and 65 kDa. The 65 kDa band has been shown previously (FSANZ 2003) to have a nearly identical mass spectrum to the 77 kDa band, indicating it is derived from the full length 77 kDa protein as a result of processing in the plant host. A minor 55 kDa band corresponds to the insecticidal tryptic core of the protein, arising as a result of processing in the plant (see above).For protein obtained from *E.coli*, a single band at approximately 77 kDa was observed..

The western blot analysis used a polyclonal goat anti-Cry3Bb1 primary antibody and a commercial horse-anti-goat horseradish peroxidase linked secondary antibody. For protein obtained from MON87411, three immunoreactive bands migrating at the expected apparent molecular weights (i.e. approximately 77 kDa, 65 kDa and 55 kDa) were observed. For protein obtained from *E.coli*, there was a single band at approximately 77 kDa.

CP4 EPSPS

For the microbially-derived protein, a single band was observed in the SDS-PAGE gel. For the plant-derived protein four bands, ranging in size from approximately 45 to approximately 90 kDa, were observed. The lowest and most prominent of these corresponded to the single band from the microbially-derived protein. The apparent molecular weight of the *E. coli*-derived protein was estimated to be 43.8 kDa while that of the prominent plant-derived protein band was 42.9 kDa. This molecular weight estimate is in good agreement with the calculated molecular weight of 47.6 kDa.

The occurrence of the other, much fainter, bands is most likely due to contaminating proteins not completely removed during the purification process. This conclusion is supported by the fact that in the Western blot analysis (below), none of these additional bands was shown to be immunoreactive.

Immunoreactivity was detected on the Western blots using a polyclonal goat anti-CP4 EPSPS primary antibody and a commercial (Thermo Scientific) rabbit-anti-goat horseradish peroxidase-linked secondary antibody. The Western blot analysis showed a single immunoreactive band, increasing in intensity with protein load, that had co-migrated in separate extracts from MON87411 and *E. coli*.

Conclusion

The Cry3Bb1 and CP4 EPSPS proteins expressed in MON87411 had the expected size and immunoreactivity.

4.1.3.2 N-terminal sequence analysis

Automated Edman degradation chemistry was performed on the major 77 and 65 kDa bands of the plant-derived Cry3Bb1 protein, and the 44 kDa band of the plant derived CP4 EPSPS protein, that had been eluted from SDS-PAGE gels.

For the Cry 3Bb1 protein, the reaction did not yield any observable sequence for the 77 kDa protein, indicating that the N-terminus was blocked. This is not uncommon and is likely due to a post-translational modification *in vivo* (see e.g. Polevoda and Sherman 2000). The amino acid analysis from the N-terminus of the truncated 65 kDa protein yielded a 15 amino acid sequence which covered positions 50 – 64 of the theoretical amino acid sequence of the full-length protein and matched the equivalent expected sequence of the Cry3Bb1 protein as deduced from the *cry3Bb1* gene sequence present in MON87411.

The data obtained for the CP4 EPSPS protein corresponded to 15 amino acids in the deduced CP4 EPSPS protein beginning at the amino acid (leucine) at position 2. The deduced amino acid at position 1 is methionine; cleavage of the N-terminal Met by methionine aminopeptidase and amino-terminal acetylation is common, particularly in eukaryotes (Polevoda and Sherman 2002; Polevoda and Sherman 2003).

4.1.3.3 MALDI-TOF tryptic mass fingerprint and intact mass analyses

Protein identification by peptide mass fingerprinting is considered reliable if the measured coverage of the sequence is 15% or higher with a minimum of five unique peptide matches with those expected from a specified enzyme digestion of the theoretical protein (Jensen et al. 1997).

For Cry3Bb1 and CP4 EPSPS, mass spectral analysis using matrix-assisted laser desorption/ionisation–time of flight mass spectrometry (MALDI–TOF MS) was performed on the trypsin-digested Cry3Bb1 77 kDa excised band and CP4 EPSPS 44 kDa band obtained by running the MON87411 protein samples on SDS-PAGE.

The peptide mapping of the MON87411 Cry3Bb1 protein identified 41 unique peptides corresponding to the masses expected to be produced by trypsin digestion, with a coverage of 73% of the amino acids expected in Cry3Bb1, which was adequate to confirm the identity of the protein. It also showed that the N-terminal Met of Cry3Bb1 had been removed and hence, that the full-length protein expressed in MON87411 comprises 652 amino acid residues. Based on the evidence from the sequence of the *cry3Bb1* gene together with N-terminal sequencing and MALDI-TOF MS, the sequence of the MON87411-derived Cry3Bb1 protein is as given in Figure 7.

```
  1 ~~M~~ANPNNRSEH DTIKVTPNSE LQTNHNQYPL ADNPNSTLEE LNYKEFLRMT EDSSTEVLDN
 61 STVKDAVGTG ISVVGQILGV VGVPFAGALT SFYQSFLNTI WPSDADPWKA FMAQVEVLID
121 KKIEEYAKSK ALAELQGLQN NFEDYVNALN SWKKTPLSLR SKRSQDRIRE LFSQAESHFR
181 NSMPSFAVSK FEVLFLPTYA QAANTHLLLL KDAQVFGEEW GYSSEDVAEF YRRQLKLTQQ
241 YTDHCVNWYN VGLNGLRGST YDAWVKFNRF RREMTLTVLD LIVLFPFYDI RLYSKGVKTE
301 LTRDIFTDPI FLLTTLQKYG PTFLSIENSI RKPHLFDYLQ GIEFHTRLRP GYFGKDSFNY
361 WSGNYVETRP SIGSSKTITS PFYGDKSTEP VQKLSFDGQK VYRTIANTDV AAWPNGKVYL
421 GVTKVDFSQY DDQKNETSTQ TYDSKRNNGH VSAQDSIDQL PPETTDEPLE KAYSHQLNYA
481 ECFLMQDRRG TIPFFTWTHR SVDFFNTIDA EKITQLPVVK AYALSSGASI IEGPGFTGGN
541 LLFLKESSNS IAKFKVTLNS AALLQRYRVR IRYASTTNLR LFVQNSNNDF LVIYINKTMN
601 KDDDLTYQTF DLATTNSNMG FSGDKNELII GAESFVSNEK IYIDKIEFIP VQL
```

Figure 7: Sequence of the full-length Cry3Bb1 protein present in MON87411 (the N-terminal Met has been removed).

The peptide mapping of the MON87411 CP4 EPSPS protein identified 23 unique peptides corresponding to the masses expected to be produced by trypsin digestion, with a coverage of 67% of the amino acids expected in CP4 EPSPS. This was adequate to confirm the identity of the protein.

Based on the evidence from the sequence of the *cp4 epsps* gene together with N-terminal sequencing and MALDI-TOF MS, the sequence of the MON87411-derived CP4 EPSPS protein is as given in Figure 8.

```
                  MLHG ASSRPATARK SSGLSGTVRI PGDKSISHRS FMFGGLASGE
TRITGLLEGE DVINTGKAMQ AMGARIRKEG DTWIIDGVGN GGLLAPEAPL DFGNAATGCR
LTMGLVGVYD FDSTFIGDAS LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP
ITYRVPMASA QVKSAVILAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR TGLILTLQEM
GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM IDEYPILAVA AAFAEGATVM
NGLEELRVKE SDRLSAVANG LKLNGVDCDE GETSLVVRGR PDGKGLGNAS GAAVATHLDH
RIAMSFLVMG LVSENPVTVD DATMIATSFP EFMDLMAGLG AKIELSDTKA A
```

Figure 8: Sequence of the CP4 EPSPS protein (minus transit peptide) present in MON87411 (the N-terminal Met has been removed).

4.1.3.4 Glycosylation status

Many eukaryotic proteins are glycoproteins that have been post-translationally modified by the addition of carbohydrate moieties (glycans) covalently linked to the polypeptide backbone.

N-glycosylated proteins are glycosylated on an asparagine residue and commonly contain an asparagine-X-serine/threonine sequence (N-X~(P)-[S/T), where X~(P) indicates any amino acid except proline (Orlando and Yang 1998). Although rare, the sequence asparagine-X-Cysteine (N-X-C) can also be an N-glycosylation site (Miletich and Broze Jr. 1990). A basic search using NetNGlyc[15] predicted four potential N-glycosylation sites in the Cry3Bb1 protein and five sites in the CP4 EPSPS protein.

Analysis of immunoaffinity-purified plant- and microbially-derived Cry3Bb1 and CP4 EPSPS proteins was done using a commercial kit (GE Healthcare) following SDS-PAGE. The kit detects carbohydrates by covalently linking biotin and visualizing for its presence using a streptavidin/peroxidase system. A glycosylated protein (transferrin) was applied to each gel as a positive control.

A visible band was obtained for transferrin while the CP4 EPSPS immunopurified proteins from both plant and microbial sources and Cry3Bb1 protein from MON87411 gave no visible bands. A band was observed in the *E.-coli*-produced Cry3Bb1 but this band was at much lower molecular weight (approximately 20 kDa) than the Cry3Bb1 band (approximately 77 kDa) and was taken to represent a naturally biotinylated protein that co-purified with the *E.coli*-produced Cry3Bb1 protein (Choi-Rhee and Cronan 2003).

These results support the conclusion that neither microbially- nor MON87411-derived Cry3Bb1 or CP4 EPSPS proteins are glycosylated.

4.1.3.5 Bioactivity

Cry3Bb1

Colorado potato beetle (CPB) larvae (≤ 30 h old) were used to measure biological activity of the Cry3Bb1 proteins produced in MON87411 and *E. coli*. The bioassay was replicated three times on separate days, each with a separate batch of larvae. Each bioassay replicate consisted of a series of six dilutions of Cry3Bb1 yielding a dose series ranging from 0.078 – 2.5 µg Cry3Bb1 protein/ml diet for the *E. coli*-produced and MON 87411-produced Cry3Bb1 and a single buffer control.

[15] http://www.cbs.dtu.dk/services/NetNGlyc/

The larvae (24/dose level) were allowed to feed individually in separate wells on an agar-based insect diet containing one of the dilutions for 7 days, after which the number of survivors at each dose level was recorded. Activity was expressed as $LC_{50,}$ µg/ml diet where LC_{50} is the concentration required to kill half of the larvae.

The LC_{50} of the MON 87411- and *E. coli*-produced Cry3Bb1 proteins was determined to be 0.77 µg/ml diet and 0.67 µg/ml diet respectively and indicated that the proteins from the two sources have equivalent activity.

CP4 EPSPS

EPSPS catalyses the transfer of the enolpyruvyl moiety of phosphoenolpyruvate to the 5-OH hydroxyl group of shikimate 3-phosphate and releases inorganic phosphate. The amount of inorganic phosphate released in the reaction is measured spectrophotometrically (660 nm) using a malachite green dye method (Lanzetta et al. 1979) and is directly related to the specific activity of the enzyme.

CP4 EPSPS from both plant- and *E. coli*-derived sources was tested for activity and values of 5.78 U/mg and 5.00 U/mg respectively were obtained. Within the accuracy of the method used, these values were considered to be indicative of equivalent functional activity of CP4 EPSPS from the two sources.

4.1.4 Safety of the Cry3B1 and CP4 EPSPS proteins

Both the Cry3Bb1 and CP4 EPSPS proteins have been previously assessed by FSANZ and determined to be safe. For Cry3Bb1, there have been two previous assessments and for the CP4 EPSPS protein there have been 14 previous assessments by FSANZ. A summary of these previous assessments is provided in Table 6. For information, the application in which the most recent detailed study or information was considered by FSANZ and is available on the FSANZ website, has been provided.

For the bioinformatic studies, which analyse sequence similarity to known protein toxins and allergens, where the Applicant provided searches (see updated studies listed below) using a more recent (and hence larger) database, the results did not alter the conclusions reached previously that neither Cry3Bb1 nor CP4 EPSPS are significantly similar to known protein toxins or allergens.

Table 6: Summary of consideration of Cry3Bb1 and CP4 EPSPS in previous FSANZ safety assessments

Consideration	Sub-section	Cry3Bb1 (link)	CP4 EPSPS (link)
Potential toxicity	History of human consumption	A548 (FSANZ 2006a)	A1071 (FSANZ 2013)
	Amino acid sequence similarity to protein toxins	This application – using database updated in 2013	This application – using database updated in 2013
	Stability to heat	A484 (FSANZ 2003)	A1071 (FSANZ 2013)
	Acute oral toxicity	A548 (FSANZ 2006a)	A1071 (FSANZ 2013)
Potential allergenicity	*In vitro* digestibility	A548 (FSANZ 2006a)	A553 (FSANZ 2006b)
	Amino acid sequence similarity to allergens	This application – using database updated in 2013	This application – using database updated in 2013

Updated studies submitted

Kang, H.T. and A. Silvanovich. 2013. Updated Bioinformatics Evaluation of the Cry3Bb1 Protein Utilizing the AD_2013, TOX_2013, and PRT_2013 Databases. **MSL0024870**. Monsanto Company (unpublished).

Kang, H.T. and A. Silvanovich. 2013. Updated Bioinformatics Evaluation of the CP4 EPSPS Protein Utilizing the AD_2013, TOX_2013, and PRT_2013 Databases. **MSL0024715**. Monsanto Company (unpublished).

Hernan, R., R. Heeren, G. Mueller, J. P. Uffman and J. Finnessy. 2011. The Effect of Heat Treatment on Cry3Bb1 Functional Activity. **MSL0023328**. Monsanto Company (unpublished).

4.1.4.1 Heat Stability of Cry3Bb1

For the heat stability of Cry3Bb1, the Applicant submitted a more recent and comprehensive study (see updated studies list) done in 2011. Cry3Bb1 protein purified from *E. coli*[16] was incubated at 0, 25, 37, 55, 75 or 95^{o} C for 15 min or 30 min. Following the treatment, the samples were then analysed by SDS - PAGE and functional activity assay.

Following SDS-PAGE, gels were stained with Brilliant Blue G-Colloidal stain and evaluated qualitatively. Heating up to 75^{o} C for either 15 min or 30 min had little effect on the intensity of the major band running at approximately 77 kDa (see Section 4.1.3.1 for a discussion of the expected molecular weight of the Cry3Bb1 protein). Heating to 95^{o} C at both time points resulted in a loss of band intensity (reflecting protein degradation) of around 90%.

For the functional activity assay, the protocol used was similar to that described for the bioactivity study (see Section 4.1.3.5). CPB larvae ($\leq$ 30 h old) were fed the protein samples. Separate bioassays were completed for the 15 min and 30 min samples each on a different day and each with a separate batch of larvae. Each bioassay consisted of seven Cry3Bb1 concentrations ranging from 0.11 – 7.0 µg Cry3Bb1 protein/ml diet for each temperature and three buffer controls. The larvae (24/dose level) were allowed to feed individually in separate wells on an agar-based insect diet containing one of the concentrations for 7 days, after which the number of survivors at each dose level was recorded. Activity was expressed as $LC_{50,}$ µg/ml diet.

The results of the functional activity assay are given in Table 7. They demonstrate that the Cry3Bb1 protein is heat labile at incubation temperatures ≥ 75^{o} C for the 15 min treatments and ≥ 55^{o} C for the 30 min treatments.

Table 7: LC_{50} values of Cry3Bb1 activity following heat treatment over 15 and 30 min

Temperature ^{o}C	15 min treatment		30 min treatment	
	LC_{50} (µg Cry3Bb1/ml diet)	% relative activity	LC_{50} (µg Cry3Bb1/ml diet)	% relative activity
0	0.83	100%	0.89	100%
25	0.99	84%	0.74	120%
37	0.86	97%	0.95	94%
55	6.7	12%	>7	<13%
75	>7	<12%	>7	<13%
95	>7	<12%	>7	<13%

Taken together, the results indicate that Cry3Bb1 is inactivated at temperatures above 75^{o} C.

[16] See Section 4.1.3 for evidence of the equivalence of the Cry3Bb1 protein derived from MON87411 and *E. coli*.

4.1.5 Bioinformatic analysis of additional ORFs created by the transformation procedure

Studies submitted:

Kang, H.T. and A. Silvanovich. 2013. Bioinformatics Evaluation of the Transfer DNA Insert in MON 87411 Utilizing the AD_2013, TOX_2013 and PRT_2013 Databases. **MSL0024883**. Monsanto Company.

Kang, H.T. and A. Silvanovich. 2013. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in MON 87411: Assessment of Putative Polypeptides. **MSL0024900**. Monsanto Company (unpublished)

This analysis, which was done using an *in silico* bioinformatic approach, was divided into two parts that considered a) the inserted T-DNA sequence from plasmid PV-ZMIR10871 and b) the junction sequence ORFs. The T-DNA analysis was done using query sequences generated by translation of the entire T-DNA, using DNAStar software in all six reading frames.

For the junction sequence ORFs the bioinformatics analysis was performed to assess the similarity to known allergens and toxins of the putative polypeptides encoded by the eight sequences obtained from the ORF analysis (refer to Section 3.4.4).

To evaluate the similarity to known allergens of proteins that might potentially be produced by translation of either the T-DNA or eight junction sequence ORFs, an epitope search was carried out to identify any short sequences of amino acids that might represent an isolated shared allergenic epitope. This search compared the sequences with known allergens in the Allergen, Gliadin and Glutenin sequence database (AD_2013), residing in the 2013 FARRP (Food Allergy Research and Resource Program) dataset within AllergenOnline[17]. The Fast Alignment Search Tool - All (FASTA) algorithm (Pearson and Lipman 1988), version 3.4t 26, was used to search the database using the BLOSUM50[18] scoring matrix (Henikoff and Henikoff 1992). No alignments with any of the eight junction sequence ORFs or the T-DNA query sequences generated an E-score[19] of ≤1e^{-5}, no alignment met or exceeded the Codex Alimentarius (Codex 2009) FASTA alignment threshold for potential allergenicity (35% identity over 80 amino acids) and no alignments of eight or more consecutive identical amino acids (Metcalfe et al. 1996) were found.

The sequences corresponding to the T-DNA translation and the eight junction sequence ORFs were also compared with sequences present in the GenBank database[20] using the FASTA algorithm. No significant similarities to any sequences in the databases (including those of known toxins) were found.

4.1.6 Conclusion

Corn line MON87411 expresses two novel proteins, Cry3Bb1 and CP4 EPSPS. For both proteins, mean levels were lowest in the grain (4.0 and 1.9 µg/g dry weight, respectively).

[17] University of Nebraska; http:www.allergenonline.org/

[18] The BLOSUM series of matrices tabulate the frequency with which different substitutions occur in conserved blocks of protein sequences and are effective in identifying distant relationships.

[19] Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Typically, alignments between two sequences will need to have an *E*-score of 1e-5 (1×10-5) or smaller to be considered to have significant homology.

[20] http://www.ncbi.nlm.nih.gov/genbank/

The highest levels of both proteins were in the whole plant samples at the V3 – V4 stage (340 and 63 µg/g dry weight, respectively) as would be expected from the high levels in both the leaves and roots at this stage.

A range of characterisation studies confirmed the identity of the Cry3Bb1 and CP4 EPSPS proteins produced in MON87411 and also their equivalence with the corresponding proteins produced in a bacterial expression system. The plant Cry3Bb1 and CP4 EPSPS proteins have the expected molecular weight (approximately 77 kDa and 43 kDa respectively), immunoreactivity, lack of glycosylation, amino acid sequence and enzyme activity.

There are no concerns regarding the potential toxicity or allergenicity of the expressed proteins, or the potential expression of any of the novel ORFs generated as a result of the transformation. Previous safety assessments of both Cry3Bb1 and CP4 EPSPS indicate that the proteins would be rapidly degraded in the stomach following ingestion and would be inactivated by heating. Additionally, updated bioinformatic studies considered in this assessment confirm the lack of any significant amino acid sequence similarity to known protein toxins or allergens. There is also no significant similarity between the encoded sequences of the novel ORFs generated as a result of the transformation and any known protein toxins or allergens.

4.2 ds DvSnf7 RNA

4.2.1 Mechanism of action

The Snf7 protein is a class E vacuolar sorting protein, and part of the Endosomal Sorting Complex Required for Transport (ESCRT-III), that is found in many organisms such as yeast, humans, mouse, fruit fly, nematode and *Arabidopsis*. Proteins marked for degradation in a cell via the lysosome are ubiquitinated and sorted into invaginations in the endosomal membrane. Snf7 is responsible for de-ubiquitination which then allows the proteins to proceed through lysosomal degradation (Teis et al. 2008; Schmidt and Teis 2011; Henne et al. 2012). Suppression of CRW DvSnf7 causes accumulation of ubiquitinated proteins, which are therefore not degraded. In turn, this leads to impairment of cell functioning in multiple tissues and eventual death of the CRW (Ramaseshadri et al. 2013).

Expression of the *DvSnf7* sequences results in the formation of a 240 bp ds*DvSnf7* RNA in the plant. It is likely the plant's own RNAi machinery responds to the presence of the dsRNA by dicing some of it into siRNAs by a pathway that is now fairly well understood (see e.g. Hammond 2005; Tomari and Zamore 2005; Siomi and Siomi 2009).

The dsDvSnf7 RNA is not immediately processed in the plant (see Section 3.7) and therefore much of it accumulates as long dsRNA. When consumed by the insect, the long dsRNA is taken up by the cells of the insect midgut and subsequently processed by the insect's RNAi machinery into 21-24-mer siRNAs (refer to Figure 9). These siRNAs act to down regulate the *DvSnf7* gene, subsequently leading to the death of the insect (Bolognesi et al. 2012). The anti-Snf7 effect in CRW has been shown to spread systemically beyond the cells of the midgut to the rest of the insect within 24 hours of ingestion of the dsRNA (Bolognesi et al. 2012). Studies have confirmed DvSnf7 transcript levels are significantly reduced in CRW as early as one day after DvSnf7 dsRNA feeding and that DvSnf7 protein levels are significantly reduced after 5 days of feeding (Bolognesi et al. 2012). It has also been shown that significant biological activity is only observed in CRW when the orally-delivered dsRNA is >60 nt. Orally delivered 21nt siRNAs are not taken up by midgut cells (Bolognesi et al. 2012), therefore any siRNAs generated by the plant cell are unlikely to contribute to the anti-Snf7 effect observed in feeding insects.

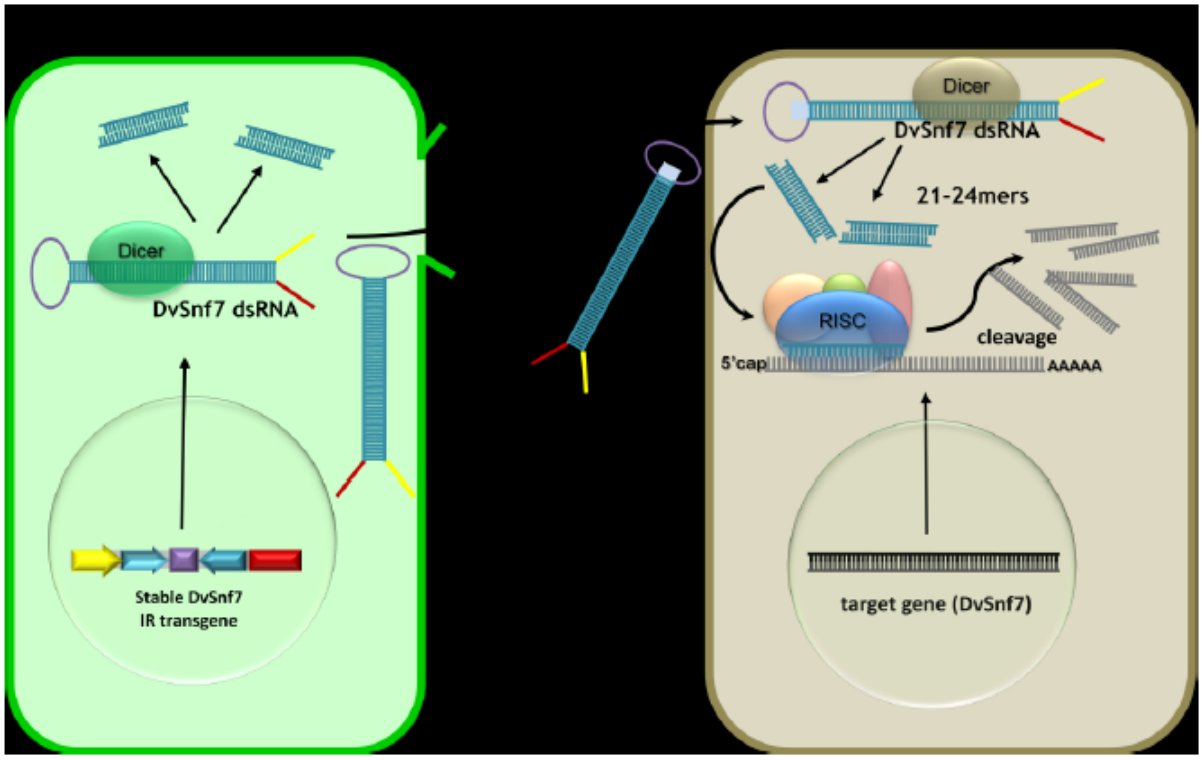


Figure 9: Schematic of RNAi suppression of the DvSnf7 gene in CRW following consumption of MON87411 tissue

4.2.1 Expression levels

The Applicant has developed an assay (based on QuantiGene technology[21]) for detecting and quantifying dsRNA transcripts (Armstrong et al. 2013). The assay work was done using the same tissue types from MON87411 collected from the field trials described in the protein expression analysis (see Section 4.1.2) and used the sense strand of the 240 nt DvSnf7 sequence as a probe. The dsDvSnf7 RNA was detected in all of the tissues sampled although the levels of expression in both pollen and grain were noted as being extremely low. The mean dsDvSnf7 RNA level was highest in whole plants at the V3 – V4 stage (0.085 µg/g dw) and lowest in grain (0.104 ng/g dw). To put the dsDvSnf7 RNA level in grain in context, the total RNA content of plant-derived food has been reported as being around 1 mg/g of tissue (Ivashuta et al. 2009). Thus, the large form of dsDVsnf7 RNA would represent less than a millionth of the total RNA in MON87411 grain.

4.2.2 Specificity

Direct feeding bioassays using 14 insect species representing 10 separate families and 4 insect Orders (Coleoptera, Hemiptera, Hymenoptera and Lepidoptera) were done to demonstrate the spectrum of activity for DvSnf7 RNAi (Bachman et al. 2013). This study demonstrated that the RNAi effect observed is highly specific to beetles within the Galerucinae subfamily of Chrysomelidae – in this case CRW and the closely related southern corn rootworm (SCR). The SCR *Snf* gene shares 98.8% sequence identity with the CRW *DvSnf7* 240 bp fragment. A number of other beetles that were tested were not susceptible and nor were any insects from the other insect Orders.

[21] http://www.panomics.com/products/gene-expression/single-plex-assay/overview

4.2.3 Safety of ingested small RNAs

FSANZ does not consider the weight of scientific evidence published to date supports the view that RNAi mediators (siRNAs, dsRNAs, miRNAs) in foods are likely to have adverse consequences for humans[22].

Numerous endogenous small RNAs and dsRNAs are present in plant and animal tissues and are a normal constituent of the human diet. Many of the small RNAs present in plant tissues have perfect or near-perfect complementarity to human and other mammalian genes (Ivashuta et al. 2009) and the same would also be true for small RNAs in animal tissues. There is no evidence to suggest the consumption of such RNAs may be associated with adverse effects in humans.

One recently published study has reported that a rice miRNA was systemically taken up via the diet and able to alter the expression of an endogenous gene in mice (Zhang et al. 2011), however little evidence has emerged since then to independently corroborate this particular finding or which demonstrates the uptake of small RNAs from food is a consistent and widespread phenomenon in human and other vertebrates. In fact, most studies that have attempted to demonstrate uptake of plant small RNAs from the diet have produced negative results (Snow et al. 2013; Dickinson et al. 2013; Witwer et al. 2013).

While RNAi has been demonstrated in some invertebrate organisms following uptake of small RNAs or dsRNAs from the diet, this is not a universal phenomenon. Invertebrates, such as certain nematode and insect species (e.g. corn rootworm), possess biological processes which facilitate the dietary uptake, systemic spread and, in some cases, amplification of small RNAs. The same cannot be said for humans and other vertebrates where equivalent processes appear to be absent or significantly diminished (Witwer and Hirschi 2014). For these species, if uptake from the diet were to occur the published evidence to date suggests the levels would be insufficient to exert a biologically relevant effect (Wang et al. 2012; Snow et al. 2013).

The evidence from therapeutic applications of RNAi to treat human disease also shows that small RNAs have only been efficacious as therapeutic agents if they are modified (through chemical modification and/or the use of carriers) to enhance their *in vivo* stability and cellular uptake (Kawakami and Hashida 2007; Burnett et al. 2011; Bramsen and Kjems 2012). In contrast, the effective uptake, systemic distribution and cellular entry of unmodified small RNAs by the oral route have proved very difficult to achieve (reviewed in Petrick et al. 2013). As a result, systemic delivery of RNAi mediators has been achieved almost exclusively by injection in mammalian studies (Witwer and Hirschi 2014).

In the absence of any evidence to suggest that the RNAi mediators produced in GM plants have different properties, or pose a greater risk, than those already naturally abundant in conventional foods, FSANZ does not consider that any additional studies (e.g. bioinformatic studies, animal studies) are necessary to demonstrate the safety of foods derived from GM plants expressing RNAi-based traits.

[22] For detailed explanation see: Response to Heinemann et al on the regulation of GM crops and foods developed using gene silencing

4.2.4 Conclusion

Corn line MON87411 expresses a 240 bp dsRNA containing sequences from the *DvSnf7* gene. The 240 bp dsRNA is detectable in a variety of tissues in MON87411, its highest expression being in whole plants around 22 days after planting (0.085 µg/g dw) and its lowest expression in the grain (0.104 ng/g dw).

A number of studies confirmed the mechanism of action of the DvSnf7 dsRNA produced in MON87411. These studies show that once ingested by the insect, the 240 bb dsRNA is taken up by the cells of the insect midgut and subsequently processed by the insect's RNAi machinery into 21-24-mer small interfering RNAs (siRNAs). These siRNAs are able to spread systemically throughout the insect and act to down regulate the *DvSnf7* gene, subsequently leading to the death of the insect.

There are no concerns regarding the safety of the DvSnf7 dsRNA in MON87411. The data provided 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 these RNAs from plant food is a widespread phenomenon in vertebrates (including humans), or if it does occur, that sufficient quantities are taken up to exert a biologically relevant effect. In addition, the level of the DvSnf7 dsRNA present in grain from MON87411is extremely low, and the anti-*DvSnf7* effect observed in corn rootworm is also highly specific to only a very small number of closely-related beetles. Grain containing the DvSnf7 dsRNA is therefore considered to be as safe for human consumption as grain derived from conventional corn varieties.

4.3 Herbicide metabolites

As part of the safety assessment it is important to establish whether the expression of a novel protein(s) is likely to result in the accumulation of any novel herbicide metabolites. If such substances are found to occur as a result of the genetic modification, then it is important to determine their potential toxicity.

The novel CP4 EPSPS protein will not result in the production of any novel metabolites of glyphosate that would not otherwise be produced in a conventional plant sprayed with glyphosate. This has been considered in previous FSANZ applications (e.g. FSANZ 2006b) and in more recent publications (see e.g. Duke 2011).

5 Compositional analyses

The main purpose of compositional analyses is to determine if, as a result of the genetic modification, any unexpected changes have 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 single constituent, 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 (e.g. solanine in potatoes).

5.1 Key components

For corn there are a number of components that are considered to be important for compositional analyses (OECD 2002). As a minimum, the key nutrients of corn grain appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, mineral and vitamin levels may be considered and international guidance also suggests that levels of the key anti-nutrients and secondary metabolites could be determined for new varieties of corn.

Corn contains a number of anti-nutrients: phytic acid, raffinose, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) and trypsin and chymotrypsin inhibitor. Only phytic acid and raffinose are considered to be biologically relevant (OECD 2002). DIMBOA is present at highly variable levels in corn hybrids and little evidence is available on either its toxicity or anti-nutritional effects, and corn contains only low levels of trypsin and chymotrypsin inhibitor, neither of which is considered nutritionally significant.

Phytic acid is considered an important anti-nutrient for animals, especially non-ruminants, since it can significantly reduce the bioavailability of phosphorus in the plant. Feed formulators add the enzyme phytase to pig and poultry diets to improve the utilisation of phosphorus. Raffinose is a non-digestible oligosaccharide and is considered an anti-nutrient because of its gastrointestinal effects (flatulence).

Secondary metabolites are defined as those natural products which do not function directly in the primary biochemical activities which support the growth, development and reproduction of the organism in which they occur. Secondary plant metabolites are neither nutrients nor anti-nutrients but are sometimes analysed as further indicators of the absence of unintended effects of the genetic modification on metabolism (OECD 2002). Characteristic metabolites in corn are furfural and the phenolic acids, ferulic acid and p-coumaric acid.

There are no generally recognised anti-nutrients in corn at levels considered to be harmful, but for the purposes of comparative assessment, the OECD has recommended considering analytical data for the content of the anti-nutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid, p-coumaric acid.

5.2 Study design and conduct for key components

Study submitted:

Klusmeyer, T. H., K. D. Miller, and R. Sorbet. 2013. Composition Analyses of Maize Forage and Grain from Glyphosate Treated MON 87411 Grown in Argentina during 2011/2012. **MSL0024658**. Monsanto Company (unpublished).

The MON87411 hybrid generation R_4F_1 (NL6169 - refer to Figure 3) was used for compositional analyses because it represents a commercial hybrid form of MON87411 that would be most applicable to food and feed use. Ideally, the comparator in compositional analyses should be the near isogenic parental line grown under identical conditions (OECD 2002). In the case of MON87411, the control was the non-GM hybrid line identified as NL6169 since this represents the closest genetic line to R_4F_1 for the purposes of comparison.

The test and control lines were grown from verified seed lots at eight field sites in Argentina[23] during the 2011-2012 growing season. Additionally, four non-GM hybrid lines were grown as reference lines at each site in order to generate tolerance ranges for each analyte. There were 20 different reference lines in total. There were four replicated plots at each site planted in a randomised complete-block design. The MON87411 plots were sprayed at the 2-4 leaf stage with glyphosate herbicide at a target rate of 0.95 kg ai/ha[24].

Grain was harvested at physiological maturity and samples were analysed for proximates, fibre (acid detergent fibre – ADF; neutral detergent fibre – NDF; total dietary fibre), fatty acids, amino acids, minerals, vitamins, anti-nutrients and secondary metabolites. The identity of harvested grain from the test and control lines was verified by event specific PCR.

Key analyte levels (proximates, fibre and minerals) for forage (harvested at R5) were also obtained but are not reported here; it is noted, however, that in the combined site analysis only the level of ash in MON87411 differed significantly from that of the control. The mean value in MON87411 was 5.57% dw while that in NL6169 was 5.95% dw but was within the 99% tolerance range and literature range (see Section 5.3 below for an explanation of these ranges).

Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods.

5.3 Analyses of key components in grain

For each analyte 'descriptive statistics' were generated i.e. a mean (least-square mean) and standard error, averaged over all sites (combined-site analysis). The values thus calculated are presented in Tables 8 – 14.

In total, 68 analyte levels were measured and carbohydrate was calculated rather than being measured i.e. a total of 69 analytes were considered. Moisture values were measured for conversion of components to dry weight, but were not statistically analysed. Sixteen analytes had more than half of the observations below the LOQ and were excluded from the statistical analysis. The data for 52 analytes were therefore analysed. This analysis used a mixed model analysis of variance. Data were transformed into Statistical Analysis Software[25] (SAS) data sets and analysed using SAS® software (SAS MIXED, version 9.2). The SAS GLM procedure was applied to all data (test, control and reference) to detect potential outliers in the dataset by screening studentized PRESS residuals[26].

The replicated sites were analysed both separately and combined across all sites (combined-site analysis). Data presented in Tables 8 – 14 represent combined-site analysis. In assessing the significance of any difference between means, a P-value of 0.05 was used (i.e. a P-value of ≥0.05 was not significant).

[23] 3 x sites at Pergamino, Buenos Aires; Hunter, Buenos Aires; Sarasa, Buenos Aires; Gahan, Buenos Aires; Los Indios, Buenos Aires; Berdier, Buenos Aires.
[24] Herbicide application rates are often expressed as 'active ingredient' per ha (ai/ha).
[25] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html
[26] A PRESS (predicted residual sum of squares) statistic provides a comparison of the predicted marginal mean and the observed mean when the predicted value is calculated without the deleted observation in question (Schabenberger 2004).

Any statistically significant differences between MON87411 and the NL6169 control were compared to the 99% tolerance interval compiled from the results for each analyte of the 20 non-GM reference lines combined across all sites, to assess whether the differences are likely to be biologically meaningful. These tolerance intervals contain, with 95% confidence, 99% of the quantities expressed in the population of commercial substances. Additionally, the results for MON87411 and NL6169 were compared to a combined literature range for each analyte, compiled from published literature for commercially available corn[27]. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within corn (Harrigan et al. 2010; Ridley et al. 2011; Zhou et al. 2011). Therefore, even if means fall outside the published range, this is not necessarily a concern.

5.3.1 Proximates and fibre

Results of the proximate and fibre analysis are shown in Table 8. Two MON87411 analyte means show a significant difference from the control – the mean protein level in MON87411 is significantly higher and the neutral density fibre (NDF) is significantly lower than the level in the control. However, both MON87411 means were within both the tolerance interval and the literature range.

Table 8: Mean (±standard error) percentage dry weight (%dw) of proximates and fibre in grain from MON87411 and NL6169

Analyte	MON87411[2] (%dw)	NL6169 (%dw)	Overall treat effect (P-value)	Tolerance interval (%dw)	Combined literature range (%dw)
Ash	1.36±0.026	1.35±0.026	0.746	1.07, 1.80	0.62 – 6.28
Protein	10.71±0.49	10.28±0.49	0.023	5.66, 15.13	6.15 – 17.26
Total Fat	3.79±0.047	3.83±0.047	0.573	1.55, 6.69	1.74 – 5.82
Carbohydrate[1]	84.13±0.51	84.53±0.51	0.068	77.72, 90.40	77.4 – 89.5
ADF	3.06±0.083	3.26±0.083	0.074	2.18, 4.98	1.82 – 11.34
NDF	8.26±0.17	8.74±0.17	0.018	6.04, 13.44	5.59 – 22.64
Total Dietary Fibre	11.50±0.20	11.82±0.20	0.247	9.83, 16.84	8.82 – 35.31

[1] Carbohydrate calculated as 100% - (protein %dw+ fat %dw + ash %dw)

[2] mauve shading represents MON87411 means that are significantly lower than the control means while orange shading represents MON87411 means that are significantly higher.

5.3.2 Fatty Acids

The levels of 22 fatty acids were measured. Of these, the following had 50% of observations below the LOQ and were therefore excluded from analysis - C8:0 caprylic, C10:0 capric, C12:0 lauric, C14:0 myristic, C14:1 myristoleic, C15:0 pentadecanoic, C15:1 pentadecenoic, C16:1 palmitoleic , C17:0 heptadecanoic, C17:1 heptadecenoic, C18:3 gamma linolenic, C20:2 eicosadienoic, C20:3 eicosatrienoic, and C20:4 arachidonic. Results for the remaining eight fatty acids are given in Table 9 and can be summarised as follows:

- There was no significant difference between the means of MON87411 and the control for palmitic, stearic, linolenic, linolenic, arachidic, eicosenoic and behenic acids.

[27] Published literature for corn incorporates references used to compile listings in the ILSI Crop Composition Database Version 4 (ILSI 2014).

- The mean level of oleic acid was significantly higher in grain of MON87411 compared with grain from the control but fell within both the tolerance interval and the combined literature range.

Table 9: Mean (±standard error) percentage composition, relative to total fat, of major fatty acids in grain from MON87411 and NL6169

Analyte	MON87411[1] (%total)	NL6169 (%total)	Overall treat effect (P-value)	Tolerance interval (%total)	Combined literature range (%total)
Palmitic acid (C16:0)	13.61±0.045	13.62±0.045	0.842	1.55, 6.69	7.94 – 20.71
Stearic acid (C18:0)	1.68±0.032	1.70 ±0.032	0.249	0.93, 2.98	1.02 – 3.40
Oleic acid (C18:1)	21.89±0.15	21.70±0.15	0.040	7.74, 50.71	17.4 – 40.2
Linoleic acid (C18:2)	60.90±0.22	61.06±0.22	0.095	33.63, 77.43	36.2 – 66.5
Linolenic acid (C18:3)	1.09±0.0093	1.09±0.0093	0.552	0.57, 1.65	0.57 – 2.25
Arachidic acid (C20:0)	0.41±0.0068	0.42±0.0068	0.571	0.21, 0.70	0.28 – 0.965
Eicosenoic acid (C20:1)	0.26±0.0018	0.26±0.0018	0.101	0.12, 0.38	0.17 – 1.917
Behenic acid (C22:0)	0.16±0.0016	0.16±0.0016	0.434	0.0065, 0.31	0.11 – 0.349

[1] orange shading represents MON87411 mean that is significantly higher than the control mean.

5.3.3 Amino acids

Levels of 18 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine.

The results in Table 10 show there was no significant difference between the control and MON87411 for the majority of analyte means but that the means for histidine and tyrosine were significantly higher in MON87411 than in NL6169. These means all fell within both the tolerance interval and the literature range.

Table 10: Mean % dw, relative to total dw, of amino acids in grain from MON87411 and NL6169

Analyte	MON87411[1] (%dw)	NL6169 (%dw)	Overall treat effect (P-value)	Tolerance interval (%dw)	Combined literature range (%dw)
Alanine	0.84±0.044	0.81±0.044	0.166	0.37, 1.24	0.439 – 1.393
Arginine	0.48±0.013	0.47±0.013	0.133	0.35, 0.66	0.119 – 0.639
Aspartate	0.68±0.028	0.66±0.028	0.068	0.42, 0.91	0.335 – 1.208
Cystine	0.21±0.0061	0.21±0.0061	0.768	0.12, 0.32	0.125 – 0.514
Glutamate	2.03±0.11	1.95±0.11	0.108	0.87, 3.02	0.965 – 3.536
Glycine	0.38±0.0099	0.38±0.0099	0.591	0.27, 0.52	0.184 – 0.539
Histidine	0.28±0.010	0.27±0.010	0.033	0.13, 0.45	0.137 – 0.434
Isoleucine	0.40±0.020	0.38±0.020	0.050	0.19, 0.56	0.179 – 0.692
Leucine	1.41±0.086	1.35±0.086	0.100	0.51, 2.14	0.642 – 2.492
Lysine	0.28±0.0057	0.27±0.0057	0.197	0.22, 0.35	0.172 – 0.668
Methionine	0.21±0.0074	0.21±0.0074	0.952	0.13, 0.28	0.124 – 0.468
Phenylalanine	0.56±0.032	0.54±0.032	0.130	0.24, 0.83	0.244 – 0.930
Proline	0.97±0.045	0.95±0.045	0.095	0.44, 1.47	0.462 – 1.632
Serine	0.48±0.023	0.47±0.023	0.095	0.25, 0.69	0.235 – 0.769
Threonine	0.37±0.015	0.36±0.015	0.131	0.22, 0.51	0.224 – 0.666
Tryptophan	0.071±0.0016	0.071±0.0016	0.805	0.053, 0.091	0.0271 – 0.215
Tyrosine	0.42±0.020	0.40±0.020	0.046	0.22, 0.58	0.103 – 0.642
Valine	0.49±0.021	0.48±0.021	0.167	0.27, 0.70	0.266 – 0.855

[1] orange shading represents MON87411 means that are significantly higher than the control means.

5.3.4 Minerals

The levels of nine minerals in grain from MON87411 and NL6169 were measured. Sodium had more than 50% of observations below the LOQ and was excluded from analysis. Results for the remaining analytes are given in Table 11 and can be summarised as follows:

- There was no significant difference between the means of MON87411 and the control for calcium, magnesium, phosphorus or potassium
- The mean levels of iron, manganese and zinc were significantly higher in grain of MON87411 compared with grain from the control but fell within both the tolerance interval and the combined literature range.
- The mean level copper was significantly lower in grain of MON87411 compared with grain from the control but fell within both the tolerance interval and the combined literature range.

Table 11: Mean levels of minerals in the grain of MON87411 and NL6169

Analyte	Unit	MON87411[1]	NL6169	Overall treat effect (P-value)	Tolerance interval	Combined literature range
Calcium	%dw	0.0031± 0.00007	0.0030± 0.00007	0.063	0.0019, – 0.0062	0.00127 – 0.02084
Copper	mg/kg dw	1.33±0.056	1.41±0.056	0.021	0.28, 3.75	0.73 – 18.5
Iron	mg/kg dw	16.84±0.41	16.33±0.41	0.013	16.55 – 24.10	10.42 – 49.07
Magnesium	%dw	0.11±0.0031	0.12±0.0031	0.657	0.086, 0.16	0.0594 – 0.194
Manganese	mg/kg dw	6.16±0.26	5.99±0.26	0.033	2.28, 12.14	1.69 – 14.30

Analyte	Unit	MON87411[1]	NL6169	Overall treat effect (P-value)	Tolerance interval	Combined literature range
Phosphorus	%dw	0.31±0.0071	0.31±0.0071	0.591	0.22, 0.43	0.147 – 0.533
Potassium	%dw	0.34±0.0061	0.35±0.0061	0.127	0.28, 0.46	0.181 – 0.603
Zinc	mg/kg dw	21.44±0.72	20.93±0.72	0.038	11.63, 36.32	6.5 – 37.2

[1] mauve shading represents MON87411 means that are significantly lower than the control means while orange shading represents MON87411 means that are significantly higher.

5.3.5 Vitamins

Levels of seven vitamins were measured. For Vitamin E, the value obtained for one replicate in one of the sites (Gahan) for one of the reference lines was flagged by the PRESS statistic as an outlier and was removed from the analysis. The outlier test procedure was reapplied to the remaining vitamin E data to detect potential outliers that were masked in the first analysis but no other values were removed.

Results are given in Table 12 and show there was no significant difference between the control and MON87411 for the majority of vitamin means but that the means for Vitamin B_1 and Vitamin B_3 were significantly lower in MON87411 than in NL6169. Both means fell within both the tolerance interval and the literature range.

Table 12: Mean weight (mg/k g dw) of vitamins in grain from MON87411 and NL6169

Analyte	MON87411[1] (mg/kg dw)	NL6169 (mg/kg dw)	Overall treat effect (P-value)	Tolerance range (mg/kg dw)	Combined literature range (mg/kg dw)
Folic acid	0.28±0.0071	0.28±0.0071	0.580	0.084, 0.56	0.147 – 1.464
Vitamin A (β-carotene)	1.29±0.058	1.38±0.058	0.145	0, 4.91	0.19 – 46.81
Vitamin B_1 (Thiamine HCl)	3.44±0.065	3.56±0.065	0.021	1.86, 5.07	1.26 – 40.00
Vitamin B_2 (Riboflavin)	1.53±0.048	1.64±0.048	0.058	0.94, 2.37	0.50 – 2.36
Vitamin B_3 (Niacin)	17.33±0.75	18.78±0.75	0.021	4.69, 42.03	10.37 – 46.94
Vitamin B_6 (Pyridoxine HCl)	6.16±0.11	6.10±0.11	0.648	3.84, 10.03	3.68 – 11.32
Vitamin E (α-tocopherol)	10.53±0.24	10.28±0.24	0.400	0, 30.69	1.5 – 68.7

[1] mauve shading represents MON87411 means that are significantly lower than the control

5.3.6 Anti-nutrients

Levels of two key anti-nutrients were measured. Results in Table 13 show that neither the mean phytic acid nor raffinose level differed significantly between MON87411 and the control.

Table 13: Mean % dw, relative to total dw, of anti-nutrients in grain from MON87411 and NL6169

Analyte	MON87411 (%dw)	NL6169 (%dw)	Overall treat effect (P-value)	Tolerance interval (%dw)	Combined literature range (%dw)
Phytic acid	0.99±0.029	0.98±0.029	0.584	0.56, 1.41	0.111 – 1.570
Raffinose	0.25±0.0081	0.24±0.0081	0.256	0, 0.45	0.020 – 0.320

5.3.7 Secondary metabolites

The levels of three secondary metabolites were measured but furfural was below the level of detection in MON87411, NL6169 and all of the reference hybrids. For the two remaining metabolites ferulic acid and p-coumaric acid (see Table 14), there was no significant difference between the control and MON87411 for either of the means.

Table 14: Mean weight (µg/g dw) of two secondary metabolites in grain from MON87411 and NL6169

Analyte	MON87411 (µg/g dw)	NL6169 (µg/g dw)	Overall treat effect (P-value)	Tolerance range (µg/g dw)	Combined literature range (µg/g dw)
Ferulic acid	1846.74±28.24	1896.61±28.24	0.110	749.39, 3421.84	291.9 – 3885.8
p-coumaric acid	148.56±2.91	148.27±2.91	0.932	0, 461.05	53.4 – 576.2

5.3.8 Summary of analysis of key components

Statistically significant differences in the analyte levels found between grain of MON87411 and the control NL6169 are summarised in Table 15. These do not raise any concerns.

Table 15: Summary of analyte levels found in grain of MON87411 that are significantly (P < 0.05) different from those found in grain of the control NL6169

Analyte	Unit	MON87411 mean[1]	NL6169 mean	diff between MON87411 & NL6169 means	diff between max and min in NL6169	MON87411 within tolerance interval?	MON87411 within literature range?
Protein	% dw	10.71	10.28	0.43	4.54	yes	yes
NDF	% dw	8.26	8.74	0.48	2.94	yes	yes
Oleic acid	% total	21.89	21.70	0.19	2.67	yes	yes
Histidine	% dw	0.28	0.27	0.01	0.12	yes	yes
Tyrosine	% dw	0.42	0.40	0.02	0.22	yes	yes
Copper	mg/kg dw	1.33	1.41	0.08	0.65	yes	yes
Iron	mg/kg dw	16.84	16.33	0.51	4.67	yes	yes
Manganese	mg/kg dw	6.16	5.99	0.17	2.68	yes	yes
Zinc	mg/kg dw	21.44	20.93	0.51	6.45	yes	yes
Vitamin B_1	mg/kg dw	3.44	3.56	0.12	0.85	yes	yes
Vitamin B_3	mg/kg dw	17.33	18.78	1.45	20.69	yes	yes

[1] mauve shading represents MON87411 means that are significantly lower than the control means while orange shading represents MON87411 means that are significantly higher.

5.4 Conclusion from compositional analyses

Detailed compositional analyses were done to establish the nutritional adequacy of grain from MON87411 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients. The levels were compared to levels in a) an appropriate non-GM hybrid line, NL6169 b) a tolerance interval compiled from results taken for 20 non-GM hybrid lines grown under the same conditions and c) levels recorded in the literature. Only 11 of the 52 analytes reported in Tables 8 – 14 deviated from the control in a statistically significant manner. However, the mean levels of all of these analytes fell within both the tolerance interval and the historical range from the literature. It is also noted that the differences between these statistically significant analyte means of MON87411 and the control means were smaller than the variation within the control. It can therefore be concluded that grain from MON87411 is compositionally equivalent to grain from conventional corn varieties.

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 well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with extensive compositional analyses of the food.

If the compositional analyses indicate biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

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 species will add little to the safety assessment and generally are not warranted (see e.g. OECD 2003; Bartholomaeus et al. 2013; Herman and Ekmay 2014). Corn line MON87411 is the result of a genetic modification designed to confer protection against corn rootworm and tolerance to the herbicide glyphosate with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of grain that have been undertaken to demonstrate the nutritional adequacy of MON87411 indicate it is equivalent in composition to conventional corn cultivars. The introduction of food from corn line MON87411 into the food supply is therefore expected to have little nutritional impact and as such no additional studies, including animal feeding studies, are required.

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