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Application to Food Standards Australia New Zealand for the Inclusion of Corn MON87411 in Standard 1.5.2 - Food Derived from Gene Technology

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ABBREVIATIONS AND DEFINITIONS[1]

~	approximately
ADF	acid detergent fiber
ANOVA	analysis of variance
AOSA	Association of Official Seed Analysts
APHIS	Animal and Plant Health Inspection Service
APS	analytical protein standards
bp	base pairs
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
bu/A	bushels per acre
bw	body weight
cDNA	complementary deoxyribonucleic acid
CEW	corn earworm
CFR	Code of Federal Regulations
CHT	ceramic hydroxyapatite
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium tumefaciens* strain CP4
CRW	corn rootworm
CTAB	hexadecyltrimethylammonium bromide
CV	coefficient of variation
DAP	days after planting
dATP	deoxyadenosine triphosphate
DDI	daily dietary intake
DEEM-FCID	Dietary Exposure Evaluation Model-Food Commodity Intake Database
DHB	2,5-dihydroxybenzoic acid
dNTP	deoxyribonucleotide
dsRNA	double stranded RNA
DTT	dithiothreitol
DvSnf7	*Snf7* gene from *Diabrotica virgifera virgifera* encoding the SNF7 subunit of the ESCRT-III complex
DvSnf7 RNA	RNA expressed from the suppression cassette that contains an inverted repeat sequence derived from the western corn rootworm (WCR; *Diabrotica virgifera virgifera*) DvSnf7 gene
DvSnf7_240	the active insecticidal RNA in MON 87411
DvSnf7_968	an *in vitro* transcribed DvSnf7 single stranded RNA
DvSnf7^{p}	partial coding sequence of the *Snf7* gene from *Diabrotica virgifera virgifera* encoding the Snf7 subunit of the ESCRT-III complex
dw	dry weight
DWCF	dry weight conversion factor

[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

ECB	European corn borer
EDV	extended diapause variant
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ESCRT	Endosomal Sorting Complex Required for Transport
EUP	experimental use permit
ETS	Excellence Through Stewardship
FA	fatty acid
FDA	U.S. Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMOC	fluorenylmethyl chloroformate
FSANZ	Food Standards Australia and New Zealand
FSE	farm scale evaluation
fw	fresh weight
GC	gas chromatography
Gb	gigabases
ha	hectare
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HT	herbicide tolerance
ILSI CCDB	International Life Sciences Institute-Crop Composition Database
IPM	integrated pest management
IRM	insect resistance management
JSC	junction sequence class
kDa	kilodalton
kg/hl	kilograms per hectoliter
LOD	limit of detection
LOQ	limit of quantitation
MEEC	maximum expected environmental concentration
MESA	4-Morpholinepranesulfonic acid - ethylenediaminetetraacetic acid - sodium acetate
MFI	median fluorescence intensity
Mg/ha	megagrams/hectare
miRNA	micro RNA
MMT	million metric tons
MOA	mode-of-action
MOE	margin of exposure
MVB	multi-vesicular bodies
n	number of samples
NCR	northern corn rootworm
NDF	neutral detergent fiber
NFDM	nonfat dry milk
NGS/JSA	Next Generation Sequencing/Junction Sequence Analysis
NHANES	National Health and Nutrition Examination Survey
NOAEL	no observable adverse effect level
NOEC	no observable effect concentration
nt	nucleotide

NTO	non-target organism
OECD	Organisation for Economic Co-operation and Development
OM	organic matter
OPA	o-phthalaldehyde
OSL	over season leaf
OSR	over season root
OSWP	over season whole plant
PBST	phosphate buffered saline containing 0.05% (v/v) Tween
PCR	polymerase chain reaction
PIP	plant incorporated protectant
Poly(A)	multiple adenosine monophosphates
PPA	Plant Protection Act
PTH-AA	phenylthiohydantoin-amino acid
QC-	negative quality control
QC+	positive quality control
RDR	root damage rating
RH	relative humidity
RISC	RNA-induced silencing complexes
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RT	room temperature
SAP	Scientific Advisory Panel
SBV	soybean variant
SCR	southern corn rootworm
SD	standard deviation
SDS	sodium dodecyl sulfate
S.E.	standard error
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
siRNA	small interfering RNA
sp.	species
TDF	total dietary fiber
T-DNA	transfer DNA
TFA	trifluoroacetic acid
TSSP	tissue-specific site pool
TTC	threshold of toxicological concern
Tz	tetrazolium
USDA	United States Department of Agriculture
UTR	untranslated region
UV	ultraviolet
v/v	volume to volume
WCR	western corn rootworm

PART 1 GENERAL INFORMATION

1.1 Applicant Details

(a) Applicant's name/s	[redacted]
(b) Company/organisation name	Monsanto Australia Limited
(c) Address (street and postal)	Level 12 / 600 St Kilda Road, Melbourne, Victoria, 3004 PO Box 6051, St Kilda Road Central, Victoria, 8008
(d) Telephone number	[redacted]
(e) Email address	[redacted]
(f) Nature of applicant's business	Technology Provider to the Agricultural and Food Industries
(g) Details of other individuals, companies or organisations associated with the application	

1.2 Purpose of the Application

This application is submitted to Food Standards Australia New Zealand by Monsanto Australia Limited on behalf of Monsanto Company.

The purpose of this submission is to make an application to vary **Standard 1.5.2** – *Food Produced Using Gene Technology* of the *Australia New Zealand Food Standards Code* to seek the addition of maize line MON 87411 and products containing maize line MON 87411 (hereafter referred to as MON 87411) to the Table to Clause 2 (see below).

Food derived from gene technology	Special requirements
Food derived from maize line MON 87411	None

1.3 Justification for the Application

1.3(a) The need for the proposed change

Monsanto Company has developed biotechnology-derived maize, MON 87411, that confers protection against corn rootworm (CRW) (*Diabrotica* spp.) and tolerance to the herbicide glyphosate. MON 87411 contains a suppression cassette that expresses an inverted repeat sequence designed to match the sequence of western corn rootworm (WCR; *Diabrotica virgifera virgifera*). The expression of the suppression cassette results in the formation of a double-stranded RNA (dsRNA) transcript containing a 240 bp fragment of the WCR *Snf7* gene (DvSnf7). Upon consumption, the plant-produced dsRNA in MON 87411 is recognized by the CRW's RNA interference (RNAi) machinery resulting in down-regulation of the targeted DvSnf7 gene leading to CRW mortality. MON 87411 also contains a *cry3Bb1* coding sequence that produces a modified *Bacillus thuringiensis* (subsp. *kumamotoensis*) Cry3Bb1 protein to protect against CRW larval feeding. In addition, MON 87411 contains the *cp4 epsps* coding sequence from *Agrobacterium* sp. strain CP4 that encodes for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, which confers tolerance to glyphosate, the active ingredient in Roundup agricultural herbicides.

1.3(b) The advantages of the proposed change over the status quo, taking into account any disadvantages

MON 87411 builds upon the current *Bt* protein-based mode-of-action (MOA) for CRW control by the addition of a new RNA-mediated MOA that offers enhanced control of target insect pests and prolonged durability of existing *Bt* technologies designed to control CRW. MON 87411 will provide benefits to growers similar to those obtained by use of existing CRW-protected maize hybrids, which include reduced need for insecticides and associated improvements in worker safety, increased yield protection, and water conservation. MON 87411 is also glyphosate tolerant and will continue to provide benefits associated with conservation tillage methods, including reduced soil erosion, reduced fuel and labour costs, improved air quality and conservation of soil moisture.

MON 87411 will not be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other biotechnology-derived products to provide protection against both above-ground and below-ground maize pests as well as tolerance to multiple herbicides. These next generation combined-trait maize products will offer broader grower choice, improved production efficiency, increased pest control durability, and enhanced grower profit potentials.

1.4 Regulatory Impact Information

1.4(a) Costs and benefits

If the draft variation to permit the sale and use of food derived from MON 87411 is approved, possible affected parties may include consumers, industry sectors and government. The consumers who may be affected are those particularly concerned about the use of biotechnology. Industry sectors affected may be food importers and exporters, distributors, processors and manufacturers. Lastly, government enforcement agencies may be affected.

A cost/benefit analysis quantified in monetary terms is difficult to determine. In fact, most of the impacts that need to be considered cannot be assigned a dollar value. Criteria would need to be deliberately limited to those involving broad areas such as trade, consumer information and compliance. If the draft variation is approved:

Consumers:

- There would be benefits in the broader availability of corn products.
- There is unlikely to be any significant increase in the prices of foods if manufacturers are able to use comingled corn products.
- Consumers wishing to do so will be able to avoid GM corn products as a result of labeling requirements and marketing activities.

Government:

- Benefit that if corn MON 87411 was detected in food products, approval would ensure compliance of those products with the Code. This would ensure no potential for trade disruption on regulatory grounds.
- Approval of corn MON 87411 would ensure no potential conflict with WTO responsibilities.
- In the case of approved GM foods, monitoring is required to ensure compliance with the labeling requirements, and in the case of GM foods that have not been approved, monitoring is required to ensure they are not illegally entering the food supply. The costs of monitoring are thus expected to be comparable, whether a GM food is approved or not.

Industry:

- Sellers of processed foods containing corn derivatives would benefit as foods derived from corn MON 87411 would be compliant with the Code, allowing broader market access and increased choice in raw materials. Retailers may be able to offer a broader range of corn products or imported foods manufactured using corn derivatives.
- Possible cost to food industry as some food ingredients derived from corn MON 87411 would be required to be labeled

1.4(b) Impact on international trade

If the draft variation to permit the sale and use of food derived from MON 87411 was rejected it would result in the requirement for segregation of any corn derived products containing MON 87411 from those containing approved corn, which would be likely to increase the costs of imported corn derived foods.

It is important to note that if the draft variation is approved, corn MON 87411 will not have a mandatory introduction. The consumer will always have the right to choose not to use/consume this product.

1.5 Assessment Procedure

Monsanto Australia is submitting this application in anticipation that it will fall within the General Procedure category.

1.6 Exclusive Capturable Commercial Benefit

This application is likely to result in an amendment to the Code that provides exclusive benefits and therefore Monsanto intends to pay the full cost of processing the application.

1.7 International and Other National Standards

1.7(a) International standards

Monsanto makes all efforts to ensure that safety assessments are aligned, as closely as possible, with relevant international standards such as the Codex Alimentarius Commission's *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* and supporting *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants* (Codex Alimentarius, 2009).

In addition, the composition analysis is conducted in accordance with OECD guidelines and includes the measurement of OECD-defined corn nutrients and anti-nutrients based on conventional commercial corn varieties (OECD, 2002a).

1.7(b) Other national standards or regulations

Monsanto has submitted a food and feed safety and nutritional assessment summary for MON 87411 to the United States Food and Drug Administration (FDA) and has also requested a Determination of Nonregulated Status for MON 87411, including all progenies derived from crosses between MON 87411 and other corn, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA).

Applications have also been and will be submitted to Canadian Food Inspection Agency (CFIA) and Health Canada (HC), Korea's Ministry of Food and Drug Safety (MFDS) for food, and Rural Development Administration (RDA) for feed use, and Japan's Ministry of Health, Labour, and Welfare (MHLW) for food use.

Regulatory submissions will be made to countries that import significant corn or food and feed products derived from countries where MON 87411 corn will be grown and have functional regulatory review processes in place. This will result in submissions to a number of additional governmental regulatory agencies including, but not limited to Ministry of Agriculture, People's Republic of China; Japan's Ministry of Agriculture, Forestry, and Fisheries, as well as to regulatory authorities in other corn importing countries with functioning regulatory systems.

PART 2 SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT

A Technical Information on the GM Food

A1 Nature and Identity of the Genetically Modified Food

A1(a) A description of the new GM organism

In its continuing efforts to provide highly effective, durable control of CRW for its customers, Monsanto Company has developed biotechnology-derived maize MON 87411 that confers protection against CRW (*Diabrotica* spp.) and tolerance to the herbicide glyphosate. MON 87411 builds upon current *Bt* protein-based CRW control technology by introducing a new MOA based on RNA-mediated gene suppression (RNAi) that offers increased control of target insect pests and will prolong the durability of existing CRW-controlling *Bt* technologies.

MON 87411 contains a suppression cassette that expresses an inverted repeat sequence designed to match the sequence in western corn rootworm (WCR; *Diabrotica virgifera virgifera*). The expression of the suppression cassette results in the formation of a double-stranded RNA (dsRNA) transcript containing a 240 bp fragment of the WCR Snf7 gene (DvSnf7). Upon consumption, the plant-produced dsRNA in MON 87411 is recognized by the CRW's RNA interference (RNAi) machinery (Tomari and Zamore, 2005; Ketting and Plasterk, 2004; Hammond, 2005) resulting in the down-regulation of the targeted DvSnf7 gene leading to CRW mortality (Bolognesi *et al.*, 2012). MON 87411 also produces a modified *Bacillus thuringiensis* (subsp. kumamotoensis) Cry3Bb1 protein to protect against CRW larval feeding. In addition, MON 87411 contains the cp4 epsps gene from *Agrobacterium* sp. strain CP4 that encodes for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, which confers tolerance to glyphosate, the active ingredient in Roundup® agricultural herbicides.

MON 87411 will provide benefits to growers similar to those obtained by use of existing CRW-protected maize hybrids, which includes reduced use of insecticides, increased yield protection, water conservation, and increased worker safety (Rice, 2004). MON 87411 is also glyphosate tolerant and will continue to provide benefits associated with conservation tillage methods, including reduced soil erosion, reduced fuel and labor costs, improved air quality and conservation of soil moisture (Hurley *et al.*, 2009; Towery and Werblow, 2010; CTIC, 2011).

MON 87411 will not be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated biotechnology-derived traits to provide protection against both above-ground and below-ground maize pests as well as tolerance to multiple herbicides. These next generation combined-trait maize products will offer the ability to maximize grower choice, improve production efficiency, increase pest control durability and improve grower profit potentials.

Applications of RNAi in Plants

Naturally occurring RNA-mediated gene suppression (RNAi) in plants has been previously documented and includes selection for soybean seed coat color (Tuteja *et al.*, 2004) and maize stalk color (Della Vedova *et al.*, 2005). In both of these instances, production of chalcone synthase was suppressed leading to significantly decreased pigmentation in soybean seed coats and maize stalks, respectively. In addition, a low glutelin rice variety has been studied and has been determined to result from production of a dsRNA and concomitant suppression of glutelin genes (Kusaba *et al.*, 2003). RNA-mediated gene suppression has also been used in a number of biotechnology-derived food crops that have previously been deregulated by the USDA or other regulatory authorities, including virus resistant papaya, squash, potato, common bean, and plum as well as a delayed ripening tomato and a soybean with altered oil composition (Parrott *et al.*, 2010). Safety assessments have been conducted (Parrott *et al.*, 2010; Petrick *et al.*, 2013) and global regulatory approvals have been obtained for products employing RNAi gene suppression.

Applications of RNAi in Insects

RNAi can also achieve gene silencing in susceptible insects following ingestion of dsRNAs (Baum *et al.*, 2007a; Whyard *et al.*, 2009; Terenius *et al.*, 2011). Insect control products can be developed utilizing RNAi sequence-specific gene silencing to suppress genes critical for insect survival. Because of this sequence-specific gene silencing, these products have the potential to selectively target a narrow group of closely related pest species and greatly reduce the likelihood of adverse effects on non-target organisms (NTOs), including those beneficial to agriculture. The spectrum of activity for DvSnf7 dsRNA has been shown to be narrow and activity is only evident in a subset of beetles within the Galerucinae subfamily of Chrysomelidae within the Order Coleoptera (Bachman *et al.*, 2013).

Mode-of-Action of the RNAi Component of MON 87411

MON 87411 contains a DvSnf7 suppression cassette that expresses an inverted repeat sequence designed to match the sequence in WCR and thereby utilizes the RNAi pathway to control CRW (*Diabrotica* spp.). The expression of the suppression cassette results in the formation of a dsRNA transcript containing a 240 bp fragment of the WCR Snf7 gene (DvSnf7). Upon consumption of MON 87411 by WCR, DvSnf7 dsRNA is recognized by the pest's RNAi machinery, resulting in the down-regulation of the targeted DvSnf7 gene leading to WCR mortality (Bolognesi *et al.*, 2012) (Figure 1).

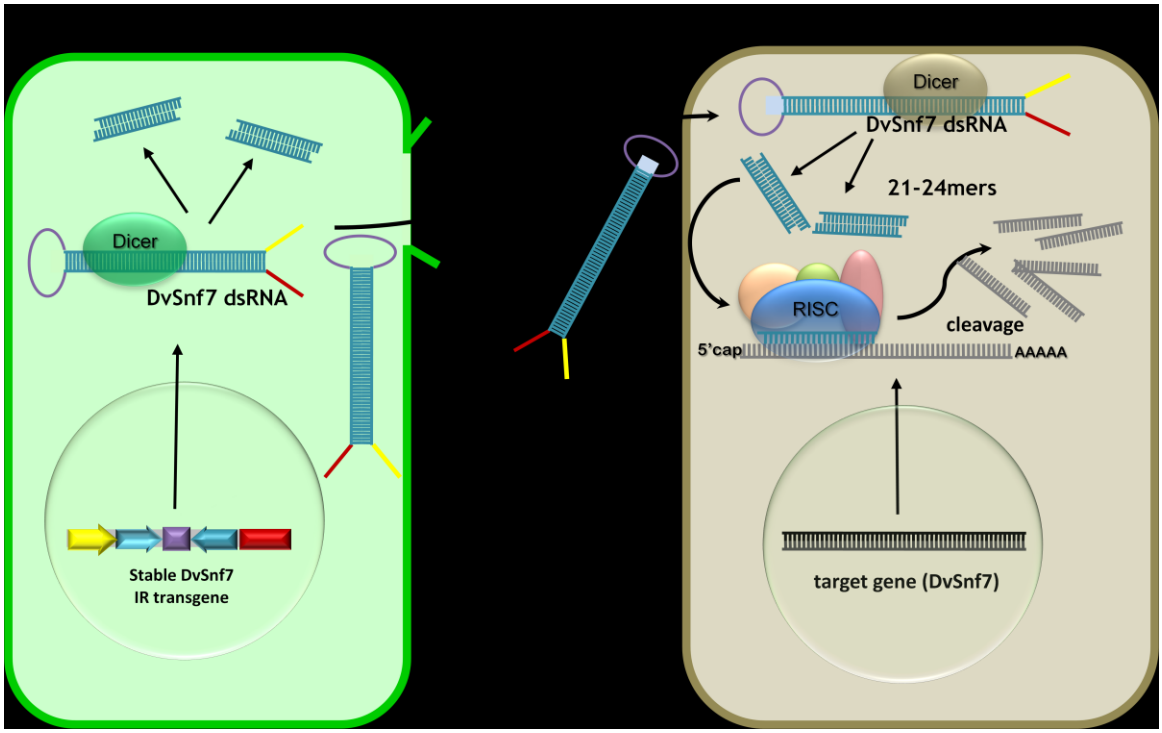


Figure 1 Diagram of MON 87411 dsRNA Oral Delivery to Suppress DvSnf7 Expression via the RNAi Pathway in CRW

The RNAi pathway is a natural process in eukaryotic organisms for the regulation of endogenous gene expression (Dykxhoorn *et al.*, 2003; Parrott *et al.*, 2010). The dsRNA molecule that activates the mechanism is first processed by a class of RNase III enzymes called Dicers into small interfering RNAs (siRNAs, ~21-25 nucleotides) (Zamore *et al.*, 2000; Hammond, 2005; Siomi and Siomi, 2009). The resulting siRNA molecules are then incorporated into multiprotein RNA-induced silencing complexes (RISC), which facilitate complementary sequence recognition and mRNA cleavage that leads to specific suppression of the target mRNA (Hammond, 2005; Tomari and Zamore, 2005). In the case of CRW that consume MON 87411, the DvSnf7 gene in CRW is suppressed.

DvSnf7 was selected as the target mRNA in CRW due to its vital cellular function that can be suppressed at relatively low concentrations when targeted by dsRNA (Baum *et al.*, 2007a). Snf7 is a class E vacuolar sorting protein and belongs to the Endosomal Sorting Complex Required for Transport (ESCRT)–III complex, which has been shown to be involved in sorting of transmembrane proteins enroute to lysosomal degradation through the endosomal-autophagic pathway in a number of organisms (Teis *et al.*, 2008; Rusten *et al.*, 2008; Lee and Gao, 2008; Vaccari *et al.*, 2009; Kim *et al.*, 2011) (Figure 2). ESCRT-III components play critical roles in distinct steps of this pathway (Roxrud *et al.*, 2010; Henne *et al.*, 2011). Data have shown that suppression of DvSnf7 in WCR leads to accumulation of ubiquitinated

proteins[2] destined for lysosomal degradation (Ramaseshadri *et al.*, 2013). Sorting of transmembrane proteins is critical to regulate signal transduction in cells and as such, suppression of this sorting through the ESCRT-III complex impairs cell homeostasis and functioning, leading to cellular death and CRW mortality. We have also shown systemic spread of the RNAi effect to tissues distal to the WCR midgut (Bolognesi *et al.*, 2012). Similar lethal phenotypes resulting from knockdown of Snf7 have been shown in Drosophila (Sweeney *et al.*, 2006) and *C. elegans* (Michelet *et al.*, 2010).

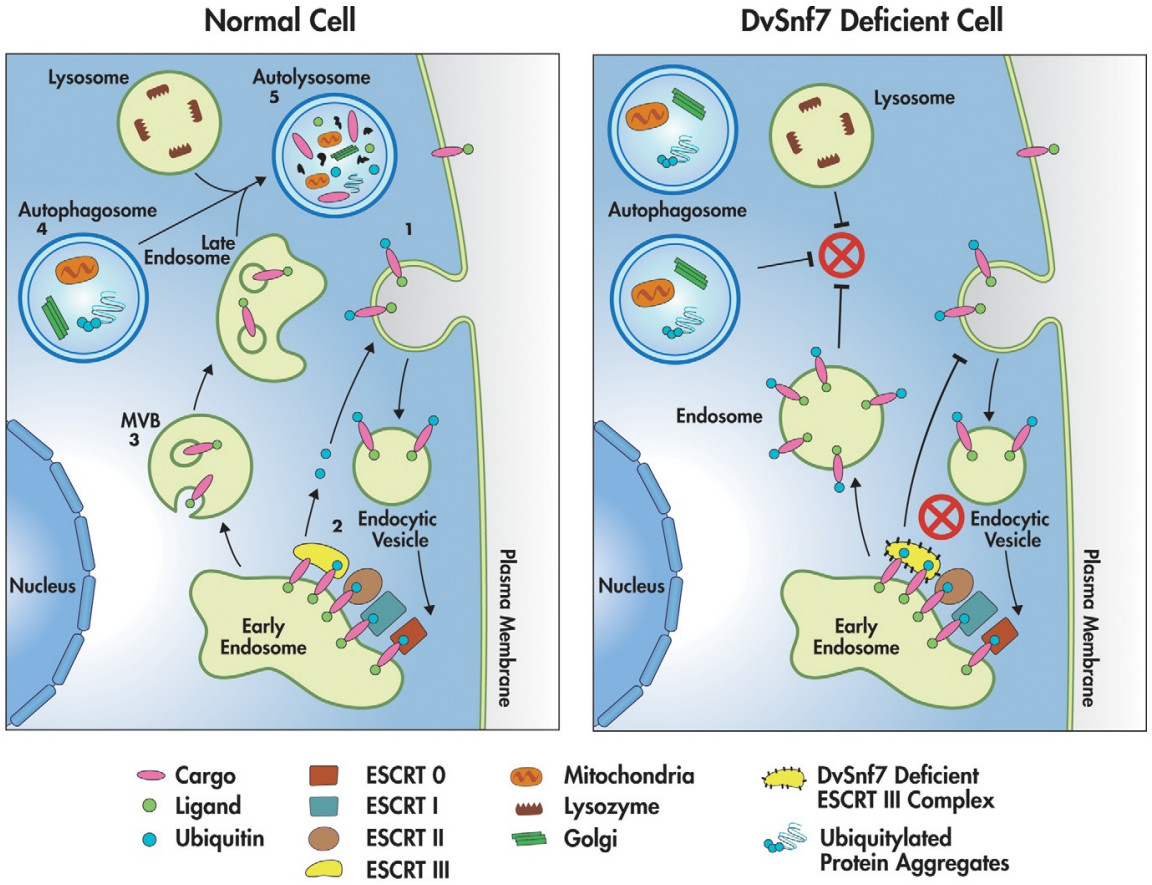


Figure 2 Model Depicting Endosomal-Autophagy Pathway Involved in Intracellular Sorting and Degradation of Receptors Along with Other Macromolecules in a Normal Cell (Left) and a DvSnf7 Deficient Cell (Right)

In the normal cell, internalization and ubiquitination of cargo proteins (1) de-ubiquitination of cargo proteins (2), biogenesis of multi-vesicular bodies (MVB) (3), formation of autophagosomes engulfing macromolecules (4), and fusion of late endosomes, autophagosome and lysosomes into autolysosomes for degradation of cargo proteins and macromolecules (5) are depicted. In the DvSnf7 deficient cell, the impairment of de-ubiquitination, accumulation of autophagosomes, and failure of fusion of endosomes,

[2] Ubiquitination is a post-translational modification of a protein in which one or more ubiquitin molecules are added to the protein (Pickart, 2001). Ubiquitins are small regulatory proteins found in all eukaryotic cells and their addition to a protein often leads to degradation of that protein. This process of ubiquitination and protein degradation allows the cell to modulate the concentration of essential proteins within that cell.

autophagosomes and lysosomes and autolysosomal activity are highlighted (Ramaseshadri *et al.*, 2013).

Induction of RNAi-mediated gene suppression in insects via an oral route of exposure requires efficient uptake of dsRNAs by midgut cells followed by suppression of the target mRNA leading to significant effects on growth, development and survival. In the case of WCR, only the relatively long dsRNA (e.g., DvSnf7 240-mer) is taken up by the insect and significant biological activity was only observed with dsRNA sequences ≥ 60 bp (Bolognesi *et al.*, 2012). Finally, several key points have been identified in demonstrating efficacy of MON 87411 against WCR: 1) oral delivery/uptake of dsRNA into WCR gut cells, 2) suppression of the targeted DvSnf7 mRNA expression followed by suppression of the production of the DvSnf7 protein, 3) systemic spread of suppression of DvSnf7 expression beyond WCR midgut tissues, and 4) growth inhibition and WCR mortality (Bolognesi *et al.*, 2012).

In addition, the spectrum of activity for the DvSnf7 dsRNA was characterized by selecting and testing insects based upon their taxonomic relatedness to the WCRW (Table 1) (Bachman *et al.*, 2013). In total, 14 insect species were tested, representing 10 families and 4 orders. Representative insects from the following orders were tested: Hemiptera, Hymenoptera, Lepidoptera and Coleoptera. In total, 7 insect species across four families in the Order Coleoptera were tested with the goal to more fully characterize the range of activity of DvSnf7 dsRNA within the Order Coleoptera. The results from these bioassays demonstrate that biological activity of DvSnf7 was only evident in a subset of beetles within the family Chrysomelidae.

Diet bioassays were conducted utilizing in vitro synthesized dsRNA, herein referred to as DvSnf7_240 dsRNA at test concentrations ranging from 500 to 5000 ng DvSnf7_240 dsRNA/mL diet. Bioassays for activity spectrum studies were designed to: (1) provide continuous exposure of the DvSnf7_240 dsRNA to each test species and (2) provide a sufficient duration of exposure to evaluate the potential effects of DvSnf7_240 dsRNA on growth, development and survival. Efficacy against the target organism WCRW, and the closely related southern corn rootworm (SCRW, *Diabrotica undecimpunctata howardi*), was characterized with larvae in 12-day concentration-response diet bioassays. Adverse effects were not detected against the Chrysomelidae species, *Leptinotarsa decemlineata*, or in representative species from the other three families tested in the order Coleoptera at exposure concentrations that will greatly exceed field exposure levels. Additionally, no adverse effects were detected in representative species from the orders Hemiptera, Hymenoptera, or Lepidoptera. Results from these bioassays demonstrate that the spectrum of activity of DvSnf7_240 dsRNA is narrow and only evident in the subfamily Galerucinae in the family Chrysomelidae within the order of Coleoptera (Table 1).

In addition, Bachman et al. (2013) confirm no activity in species that lack ≥ 21nt sequence matches to DvSnf7 and also demonstrate the 240 nt sequence of Snf7 was shown to diverge rapidly across insects within the Chrysomelidae and the divergence of the sequence and lack of ≥ 21 nt contiguous matches to DvSnf7 was evident at the subfamily and tribe level (Table

2). Therefore, it is considered that arthropods outside a subset of the Chrysomelidae family would not be susceptible to the DvSnf7 dsRNA.

Along with the sequence specificity, there exist additional barriers to the oral toxicity in insects. These include potential degradation of the dsRNA prior to ingestion as well as the inherent sensitivity of the organism to ingested dsRNA (Whyard *et al.*, 2009). For example, recent studies on the tarnished plant bug (*Lygus lineolaris*, Hemiptera) demonstrated that endonucleases present in saliva rapidly degrade dsRNA creating a barrier to an RNAi effect in this species by oral delivery of dsRNA (Allen and Walker, 2012). In addition, as summarized in a recent review by Huvenne and Smagghe (Huvenne and Smagghe, 2010), insects display a wide range of sensitivities to ingested dsRNA, with the order Coleoptera demonstrating significantly greater sensitivity than other insect orders. For example, the order Lepidoptera has demonstrated variable susceptibility to ingested dsRNA and high concentrations are required to elicit a response in this order relative to Coleopterans (Huvenne and Smagghe, 2010; Terenius *et al.*, 2011).

Table 1 Susceptibility to DvSnf7_240 dsRNA in Laboratory Bioassays

Order	Family	Subfamily	Species Tested	LC_{50} value or Concentration Tested (ng DvSnf7/mL or g diet)	Activity
Hemiptera	Anthocoridae	Anthocorinae	*Orius insidiosus*	5000	-
Hymenoptera	Eulophidae	Entedoninae	*Pediobius foveolatus*	3000	-
	Pteromalidae	Pteromalnae	*Nasonia vitripennis*	5000	-
Lepidoptera	Noctuidae	Noctuinae	*Spodoptera frugiperda*	500	-
		Heliothinae	*Helicoverpa zea*	5000	-
	Crambidae	Pyraustinae	*Ostrinia nubilalis*	5000	-
	Bombycidae	Bombycinae	*Bombyx mori*	5000	-
Coleoptera	Carabidae	Harpalinae	*Poecilus chalcites*	5000	-
	Coccinellidae	Coccinellinae	*Coleomegilla maculata*	3000	-
		Epilachninae	*Epilachna varivestis*	3000	-
	Tenebrionidae	Tenebrioninae	*Tribolium castaneum*	5000	-
	Chrysomelidae	Chrysomelinae	*Leptinotarsa decemlineata*	5000	-
		Galerucinae	*Diabrotica undecimpunctata howardi*	1.2	+
			Diabrotica virgifera	4.4	+

Table 2 Bioinfomatics Data of *Snf7* Orthologs from Selected Coleoptera and Associated Sensitivity to DvSnf7 dsRNA (Adapted from Bachman *et al*., 2013)

Order, Family and Subfamily	Species	Percent identity to Dvsnf7 dsRNA	No. 21 nt matches	Activity
Coleoptera (Chrysomelidae, Galerucinae)	**WCR** (*Diabrotica virgifera virgifera)*	100.0	221	+
	SCR (*Diabrotica undecimpunctata howardi)*	98.8	186	+
	Acalymma vittatum	95.0	69	+[2]
	Ceratoma trifurcata	90.8	18	+[2]
	Galerucella calamriensis	90.8	3	+[2]
Coleoptera (Chrysomelidae, Chrysomelinae)	**CPB** (*Leptinotarsa decemlineata*)	78.3	0, (14 nt)[1]	-
	Chrysolina quadrigemina	82.1	0, (19 nt)[1]	-[2]
	Microtheca ochroloma	79.6	0, (19 nt)[1,3]	-[2]
Coleoptera (Tenebrionidae, Tenebrioninae)	*Tribolium castaneum*	72.1	0, (11 nt)[1,3]	-

[1] Longest contiguous sequence
[2] These species are difficult to maintain and bioassay in the laboratory, so indirect diet bioassays using their *Snf7* orthologs were conducted in order to evaluate the potential toxicity of DvSnf7 dsRNA against these species.

Modes-of-Action of the CP4 EPSPS and Cry3Bb1 Proteins

MON 87411 contains the identical CP4 EPSPS protein that is expressed in MON 88017 maize (USDA-APHIS Petition No. 04-125-01p) and numerous other Roundup Ready® crops (maize, cotton, soybean, canola, alfalfa, sugar beet). The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgette *et al.*, 1996). In MON 87411, as in other Roundup Ready plants, aromatic amino acids and other metabolites necessary for plant growth and development are produced by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette *et al.*, 1996).

MON 87411 also contains an expression cassette that codes for the same Cry3Bb1 protein as the expression cassette that is present in MON 88017 maize (USDA-APHIS Petition No. 04-125-01p) that was granted non-regulated status by the USDA-APHIS in 2006 (USDA-APHIS, 2013). The amino acid sequence deduced from the Cry3Bb1 expression cassettes of MON 87411 and MON 88017 is also 99.8% identical to the deduced amino acid sequence for Cry3Bb1 protein in MON 863 (USDA-APHIS Petition No. 01-137-01p) that was granted non-regulated status by the USDA-APHIS in 2002 (USDA-APHIS, 2013). The use of Bt-containing crops in U.S. agriculture has been widespread and the mode-of-action (i.e., solubilization of Cry protein, processing to the active form, binding to midgut receptors and insertion of the toxin into cellular membranes) and specificity of Bt proteins has been studied extensively and is well understood (Gill *et al.*, 1992; Whalon and Wingerd, 2003).

A1(b) Name, number or other identifier of each new line or strain

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants" MON 87411 has been assigned the unique identifier MON-87411-9.

A1(c) The name the food will be marketed under (if known)

Maize containing the transformation event MON 87411 will be produced in North America. There are currently no plans to produce this product in Australia and New Zealand. A commercial trade name for the product has not been determined at the time of this submission and will be available prior to commercial launch of the product in North America.

A1(d) The types of products likely to include the food or food ingredient

Maize is widely used for a variety of food and feed purposes, and it is intended that MON 87411 will be utilized in the same manner and for the same uses as conventional maize. Maize grain and its processed products are consumed in a multitude of human food and animal feed products. Maize forage (as silage) is extensively consumed as an animal feed by ruminants.

A2 History of Use of the Host and Donor Organisms

A2(a) Description of all donor organism(s)

A2(a)(i) Common and scientific names and taxonomic classification

MON 87411 contains a suppression cassette that expresses an inverted repeat sequence designed to match the sequence of western corn rootworm (WCR; *Diabrotica virgifera virgifera*). The expression of the suppression cassette results in the formation of a double-stranded RNA (dsRNA) transcript containing a 240 bp partial fragment of the WCR Snf7 gene (DvSnf7). Upon consumption, the plant-produced dsRNA in MON 87411 is recognized by the CRW's RNA interference (RNAi) machinery resulting in down-regulation of the targeted DvSnf7 gene leading to CRW mortality. MON 87411 also contains a cry3Bb1 coding sequence that produces a modified *Bacillus thuringiensis* (subsp. *kumamotoensis*) Cry3Bb1 protein to protect against CRW larval feeding. In addition, MON 87411 contains the cp4 epsps coding sequence from *Agrobacterium* sp. strain CP4 that encodes for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, which confers tolerance to glyphosate, the active ingredient in Roundup agricultural herbicides.

The donor organism, Agrobacterium sp. strain CP4, was isolated based on its tolerance to glyphosate brought about by the production of a naturally glyphosate-tolerant EPSPS protein (Padgette *et al.*, 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection (ATCC) as an Agrobacterium species. This identification was made based on morphological and biochemical characteristics of the isolate and its similarity to a reference strain of Agrobacterium. The taxonomy of *Agrobacterium* sp. is:

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rhizobiales

Family: Rhizobiaceae

Genus: *Agrobacterium*

The donor organism for cry3Bb1, *Bacillus thuringiensis*, is used commercially in the U.S. to produce microbial-derived products with insecticidal activity. The taxonomy of *Bacillus thuringiensis* is:

Kingdom:Eubacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: *Bacillus*

Species: *thuringiensis*

Subspecies: *kumamotoensis*

The sequence present in the DvSnf7 suppression cassette in MON 87411 was designed to partially match the gene present in western corn rootworm (WCR: Diabrotica virgifera virgifera) encoding the SNF7 subunit of the ESCRT-III complex (Babst *et al.*, 2002). Only a portion of the similar sequence in western corn rootworm was utilized in MON 87411 to induce the RNAi mechanism in CRW.

The partical fragment of the WCR *Snf7* gene (DvSnf7) contained in MON 87411 is designed to match *Diabrotica virgifera virgifera*. The taxonomy of *Diabrotica virgifera virgifera* is:

The taxonomy of western corn rootworm (WCR; *Diabrotica virgifera virgifera*) is:

Domain: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Coleoptera

Family: Chrysomelidae

Genus: *Diabrotica*

Species: *virgifera*

Subspecies: *virgifera*

A2(a)(ii) Information on pathogenicity, toxicity, allergenicity

There is no evidence of human or animal pathogenicity for any of the donor organisms of the coding and non-coding DNA sequences present in MON 87411. DNA has always been present in feed and food and upon consumption, is quickly degraded to nucleic acids by nucleases present in the gastrointestinal tract of humans and animals. According to the U.S. FDA (U.S. FDA, 1992), nucleic acids which are present in the cells of every living organism, do not raise concerns as a component of food and are generally recognized as safe (GRAS). Results from an International Life Sciences Institute (ILSI) workshop on safety considerations of DNA in food were reported (Jonas *et al.*, 2001) and confirmed that: 1) all DNA, including recombinant DNA, is composed of the same four nucleotides; 2) there are no changes to the chemical characteristics or the susceptibility to degradation by chemical or enzymatic hydrolysis of recombinant DNA as compared to non-recombinant DNA; and 3) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome. Additionally, the European Food Safety Authority (EFSA) has reported that a large number of experimental studies have shown that recombinant DNA consumed by livestock has not been subsequently detected in tissues, fluids, or edible products of these farm animals (EFSA, 2007).

A2(a)(iii) History of use of the organism in food supply or human exposure

The donor organism for cry3Bb1, *Bacillus thuringiensis*, has been used commercially in the United States since 1958 to produce microbial-derived products with insecticidal activity. The extremely low mammalian toxicity of *Bt*-based insecticide products has been demonstrated in numerous safety studies, and there are no confirmed cases of allergic reactions to Cry proteins in applicators of microbial-derived *Bt* products during 50 years of

use. Applications of sporulated *Bt* have a long history of safe use for pest control in agriculture, especially in organic farming (Cannon, 1993; WHO, 1999; U.S. EPA, 1988). Microbial pesticides containing *Bt* Cry3 proteins have been used for more than 30 years and subjected to extensive toxicity testing showing no adverse effects to human health (U.S. EPA, 2005; U.S. EPA, 2001a; Baum *et al.*, 1999; Betz *et al.*, 2000; Mendelsohn *et al.*, 2003; McClintock *et al.*, 1995). The history of safe use of *Bacillus thuringiensis* (subspecies *kumamotoensis*) has been previously reviewed by FSANZ as a part of the safety assessment of MON 88017 and MON 863 maize which encodes cry3Bb1. MON 88017 and MON 863 were approved by FSANZ.

The donor organism for cp4 epsps, *Agrobacterium* sp. strain CP4, is not known for human or animal pathogenicity, and is not commonly allergenic (FAO-WHO, 1991). The history of safe use of Agrobacterium sp. strain CP4 has been previously reviewed by FSANZ as a part of the safety assessment of MON 40-3-2, MON 89788 and MON 87705 (soybean), NK603, MON 88017 and MON 87427 (maize), RT73 and MON 88302 (canola), J101&J163 and KK179 (alfalfa), MON 1445 and MON 88913 (cotton), which encodes cp4 epsps. All of these events have been approved by FSANZ.

The sequence present in the DvSnf7 suppression cassette in MON 87411 was designed to partially match the gene present in western corn rootworm (WCR: *Diabrotica virgifera virgifera*) encoding the SNF7 subunit of the ESCRT-III complex (Babst *et al.*, 2002). Only a portion of the similar sequence in western corn rootworm was utilized in MON 87411 to induce the RNAi mechanism in CRW. Based on the ubiquitous nature of RNAi suppression utilizing endogenous dsRNAs in a wide variety of plant species consumed by humans and animals, demonstration of the specificity of DvSnf7 suppression in CRW (Bachman *et al.*, 2013), the long history of safe consumption of RNA from a range of sources, and the apparent lack of toxicity or allergenicity of dietary RNA; the DvSnf7 RNAi suppression sequence used in MON 87411 poses no observed risks to humans or animals. Further details can also be found in Section A1(a).

A2(b) Description of the host organism

A2(b)(i) Phenotypic information

Maize (*Zea mays* L.) is a member of the tribe Maydae, which is included in the subfamily Panicoideae of the grass family Gramineae.

Family - Gramineae
- Subfamily - Panicoideae
  - Tribe - Maydae
    - Western Hemisphere:
      - Genus - *Zea*
        - A. Subgenus - *Luxuriantes*
          1. *Zea luxurians* (2n = 20)
          2. *Zea perennis* (2n = 40)
          3. *Zea diploperennis* (2n = 20)
        - B. Subgenus - *Zea*
          1. *Zea mays* (2n = 20)
             Subspecies
             1. *Z. mays parviglumis* (2n = 20)
             2. *Z. mays huehuetenangensis* (2n = 20)
             3. *Z. mays mexicana* (Schrad.) (2n = 20)

The genera included in the tribe Maydae include *Zea* and *Tripsacum* in the Western Hemisphere, and *Coix*, *Polytoca*, *Chionachne*, *Schlerachne*, and *Trilobachne* in Asia. Although some researchers have implicated the Asian genera in the origin of maize, the evidence for them is not as extensive and convincing as for the genera located in the Western Hemisphere.

The genus *Zea* includes two sub-genera: *Luxuriantes* and *Zea*. Maize (*Zea mays* L.) is a separate species within the subgenus *Zea,* along with three subspecies. All species within the genus *Zea*, except maize, are different species of teosinte. Until recently, the teosinte species were included in the genus *Euchlaena* rather than the genus *Zea*.

Maize is grown in nearly all areas of the world and is the largest cultivated crop in the world followed by wheat (*Triticum* sp.) and rice (*Oryza sativa* L.) in total global metric ton production. In 2012, maize was planted globally on ~174 million hectares (ha) with a total grain production of an estimated 854 million metric tons (MMT) (USDA-FAS, 2013). The top five production regions in 2012 were: USA (274 MMT), China (208 MMT), Brazil (73 MMT), EU-27 (55 MMT), and Argentina (27 MMT) (USDA-FAS, 2013). In the U.S., maize is grown in almost every state and in 2012, its production value of over $77 billion was the highest of any crop (USDA-NASS, 2013).

In industrialized countries maize has two major uses: (1) as animal feed in the form of grain, forage or silage; and (2) as a raw material for wet- or dry-milled processed products such as high fructose maize syrup, oil, starch, glucose, dextrose and ethanol. By-products of the wet- and dry- mill processes are also used as animal feed. These processed products are used as ingredients in many industrial applications and in human food products. Most maize produced in industrialized countries is used as animal feed or for industrial purposes, but maize remains an important food staple in many developing regions, especially sub-Saharan

Africa and Central America, where it is frequently the mainstay of human diets (Morris, 1998).

Maize is a very familiar plant that has been rigorously studied due to its use as a staple food/feed and the economic opportunity it brings to growers. The domestication of maize likely occurred in southern Mexico between 7,000 and 10,000 years ago (Goodman, 1988). While the putative progenitor species of maize have not been recovered, it is likely that teosinte played an important role in contributing to the genetic background of maize. Although grown extensively throughout the world, maize is not considered a persistent weed or a plant that is difficult to control. Maize, as we know it today, cannot survive in the wild because the female inflorescence (the ear) is covered by a husk thereby restricting seed dispersal, it has no seed dormancy, and is a poor competitor in an unmanaged ecosystem. The transformation from a wild, weedy species to one dependent on humans for its survival most likely evolved over a long period of time through plant breeding by the indigenous inhabitants of the Western Hemisphere. Today, virtually all the maize grown in the U.S. is a hybrid, a production practice that started in the 1930's (Wych, 1988). Maize hybrids are developed and used based on the positive yield increases and plant vigor associated with heterosis, also known as hybrid vigor.

Conventional plant breeding results in desirable characteristics in a plant through the unique combination of genes already present in the plant. However, there is a limit to genetic diversity with conventional plant breeding. Biotechnology, as an additional tool to conventional breeding, offers access to greater genetic diversity than conventional breeding alone, resulting in expression of highly desirable traits that are profitable to growers.

A2(b)(ii) How the organism is propagated for food use

Maize is wind-pollinated, and the distances that viable pollen can travel depend on prevailing wind patterns, humidity, and temperature. Pollen is shed from the tassel and is viable for approximately 10 to 30 minutes as it is rapidly desiccated in the air (Kiesselbach, 1980). Maize plants shed pollen for up to 14 days.

The reproductive phase begins when one or two auxiliary buds, present in the leaf axils, develop and form the pistillate inflorescence of female flower. The auxiliary bud starts the transformation to form a long 'cob' on which the flowers will be borne. From each flower a style begins to elongate towards the tip of the cob in preparation for fertilization. These styles form long threads, known as silks. The base of the silk is unique, as it elongates continuously until fertilization occurs. Styles may reach a length of 30 centimetres, the longest known in the plant kingdom. Individual maize kernels, or fruit, are unique in that mature seed is not covered by floral bracts (glumes, lemmas, and paleas) as in most other grasses, but rather the entire structure is enclosed and protected by large modified leaf bracts, collectively referred to as the ear. The mature female flowers will remain ready for fertilization for up to two weeks, at which point if fertilization has not occurred, the nucleus will de-organize and fertilization will no longer be possible.

The pollen of maize, a protandrous plant, matures before the female flower is receptive. This may have been an ancient mechanism to ensure cross-pollination, but is no longer

considered conducive to modern agricultural practices. However, decades of conventional selection and improvement have produced many maize varieties with similar maturities for both male and female flowers, to ensure seed set for agricultural purposes.

Under natural conditions, maize reproduces only by seed production. Pollination occurs with the transfer of pollen from the tassels to the silks of the ear. About 95% of the ovules are cross-pollinated and about 5% are self-pollinated, although plants are completely self-compatible. Maize, as a thoroughly domesticated plant, has lost all ability to disseminate its seeds and relies entirely on the aid of man for its distribution.

A2(b)(iii) What part of the organism is used for food

Maize has been a staple of the human diet for centuries, and its processed fractions are consumed in a multitude of food and animal feed products. In the U.S. the demand for maize is driven by the demand for feed and fuel. In 2009, animal feed accounted for almost 43% of maize consumption followed by over 32% for ethanol, and approximately 10% for food and industrial uses with the remainder being exported (NCGA, 2013). Global demand for animal feed rose 20% during 2008. Expanding economies in China, India and Brazil between 1990 and 2006 have led to greater consumption of meat and dairy products (von Braun, 2007).

Food uses include sweet corn, popcorn, and processed field maize, which are all varieties/hybrids of Zea mays subsp. mays. The majority of maize used for food and industrial purposes is processed by wet milling to produce starch and sweetener products (e.g., high fructose corn syrup) for use in foodstuffs. Non-food products such as industrial starches, maize gluten feed and maize gluten meal are also manufactured through the wet mill process (May, 1987). The primary products derived from the dry milling process are corn meal, corn flour, and ethanol.

Due to its high starch and low fiber contents, maize is considered a valuable energy source in animal feed for livestock such as cattle, pigs and poultry. Whole maize is usually ground and mixed with a high-protein feed compound and with vitamin and mineral supplements to balance the ratio according to the nutritional requirements of the animals being fed (Leath and Hill, 1987). Maize is also used for processing and the production of derivatives, which have a wide range of food, feed and industrial applications. Some of the processed fractions are used for animal feed, such as maize gluten, a resource that is rich in maize protein. Ethanol production from the dry mill process provides dried distiller's grain solubles (DDGS) which are another source of animal feed (RFA, 2010).

A2(b)(iv) Whether special processing is required to render food safe to eat

Maize grain contains 82% endosperm, 12% germ, 5% bran, and 1% tip cap. In addition, 2.2% of the bran fraction is made up of crude fiber (Earle and Curtis, 1946; Perry, 1988). Food uses include sweet corn, popcorn, and processed field maize, which are all varieties/hybrids of Zea mays subsp. mays.

Maize processing methods include wet milling, dry milling, and fermentation. The milling process separates the maize kernel into three basic parts; endosperm, pericarp, and the germ (Watson, 1988).

Products from wet milling: The majority of the maize used for food and industrial purposes is processed by wet milling to produce starch and sweetener products for use in foodstuffs. Starch is used as a food ingredient in: dairy and ice cream; batters and breading; baked goods; soups, sauces and gravies; salad dressings; meat and poultry; confections; and, in drinks. Starch can also be converted to a variety of sweetener and fermentation products including high fructose maize syrup and ethanol (Watson, 1988).

Products from dry milling: The primary food products derived from the dry milling process are maize grits, maize meal, and maize flours. Maize grits are derived from endosperm of the maize kernel, with less than 1 % oil content. Maize grits are consumed in the U.S. as side dish for breakfast. Maize meal, however, has larger particles than maize grits and is often enriched with thiamine, riboflavin, niacin, and iron to produce baked products such as maize bread and muffins. Maize flour consists of fine endosperm particles, and is often used as a binder in processed meats, as well as in producing several snack foods (Rooney and Serna-Saldivar, 1987).

Products from fermentation: Starch produced from the wet milling process can also be used in producing ethanol and distilled beverages through fermentation (Rooney and Serna-Saldivar, 1987).

A2(b)(v) The significance to the diet in Australia and New Zealand of the host organism

Maize has been a staple of the human diet for centuries, and its processed fractions are consumed in a multitude of food. Estimates of maize consumption are available from the WHO Global Environmental Monitoring System - Food Contamination Monitoring and Assessment Programme (GEMS/Food) (www.who.int/foodsafety/chem/gems). The GEMS/Food programme has developed 13 Cluster Diets which are considered to be representative of the major food consumption patterns exhibited by regional and cultural groups around the world. Australia is included in Cluster M, along with United States and Canada and several other countries

A3 The Nature of the Genetic Modification

A3(a) Method used to transform host organism

MON 87411 was developed through *Agrobacterium*-mediated transformation of immature maize embryos based on the method described by Sidorov and Duncan (2009), utilizing PV-ZMIR10871. Immature embryos were excised from a post-pollinated maize ear of LH244. After co-culturing the excised immature embryos with Agrobacterium carrying the plasmid vector, the immature embryos were placed on selection medium containing glyphosate and carbenicillin disodium salt in order to inhibit the growth of untransformed plant cells and excess Agrobacterium. Once transformed callus developed, the callus was placed on media conducive to shoot and root development. The rooted plants (R0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment. As demonstrated in this petition, the use of disarmed Agrobacterium tumefaciens strain ABI, a designated plant pest, as the transformation vector has not imparted plant pest characteristics to MON 87411.

The R_0 plants generated through the transformation process described above had already been exposed to glyphosate in the selection medium and demonstrated glyphosate tolerance. The R_0 plants self-pollinated to produce R_1 seed and R_1 plants were evaluated for the presence of the T-DNA via quantitative polymerase chain reaction (PCR) analysis. The R_1 plants homozygous for the T-DNA were selected for further development and their progenies were subjected to further molecular and phenotypic assessments. As is typical of a commercial event production and selection process, hundreds of different transformation events (regenerants) were generated in the laboratory using PV-ZMIR10871. After many months of careful selection and evaluation of these hundreds of events in the laboratory, greenhouse and field, MON 87411 was selected as the lead event based on superior agronomic, phenotypic, and molecular characteristics. Studies on MON 87411 were initiated to further characterize the genetic insertion and the expressed products, and to establish the food, feed, and environmental safety relative to commercial maize. The major steps involved in the development of MON 87411 are depicted in Figure 3.

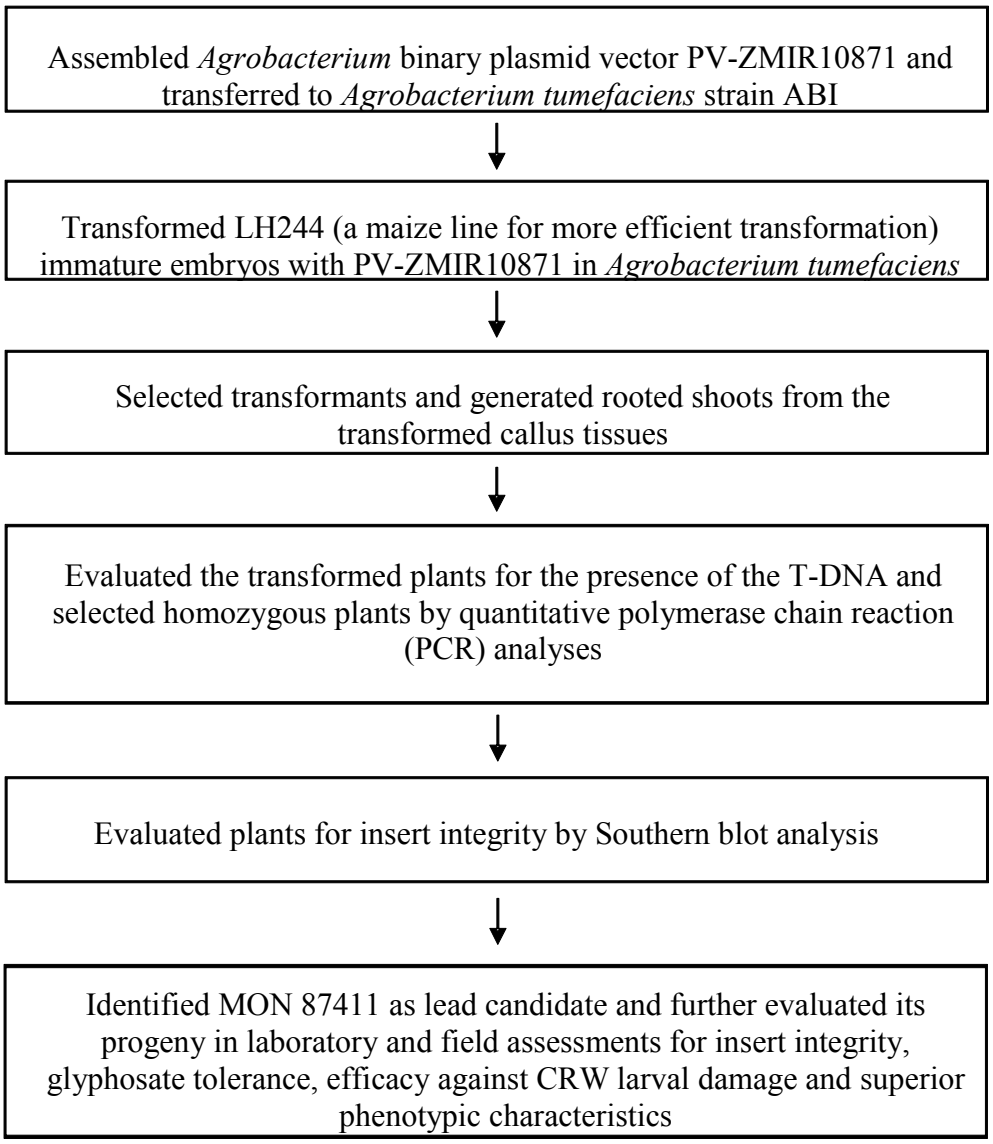


Figure 3 Schematic of the Development of MON 87411

A3(b) Intermediate hosts (*e.g.* bacteria)

A disarmed strain of *Agrobacterium tumefaciens* was the intermediate host used to transfer the plasmid PV-ZMIR10971 into maize cells. PV-ZMIR10871 contains one T-DNA containing the *DvSnf7* surpression cassete , the full *cp4 epsps* expression cassette and *cry3Bb1* expression cassette. Following transformation, self-pollination, breeding, and segregation methods were used to produce MON 87411 .

A3(c)(i) Gene construct including size, source and function of all elements

MON 87411 was developed through *Agrobacterium tumefaciens*-mediated transformation of maize immature embryos from line LH244 utilizing PV-ZMIR10871.

PV-ZMIR10871

PV-ZMIR10871 was used in the transformation of maize to produce MON 87411 and its plasmid map is shown in Figure 6 at Section A3(c)(ii). The elements included in this plasmid vector are described in Table 3. PV-ZMIR10871 is approximately 16.5 kb and contains one T-DNA that is delineated by Left and Right Border regions. The T-DNA contains the DvSnf7 suppression cassette, the *cry3Bb1* expression cassette, and the *cp4 epsps* expression cassette. The DvSnf7 suppression cassette is regulated by the *e35S* promoter from the 35S RNA of cauliflower mosaic virus (CaMV), the heat shock protein 70 (*Hsp70*) intron from *Zea mays*, and the 3' untranslated sequence of the *E9* gene from *Pisum sativum*. The *cry3Bb1* expression cassette is regulated by the *pIIG* promoter from *Zea mays*, the chlorophyll a/b binding protein (CAB) leader from *Triticum aestivum*, the *Ract1* intron from *Oryza sativa*, and the heat shock protein 17 (*Hsp17*) 3′ untranslated region from *Triticum aestivum*. The *cp4 epsps* expression cassette is regulated by the *TubA* promoter from *Oryza sativa*, the *TubA* leader from *Oryza sativa*, the *TubA* intron from *Oryza sativa*, the *CTP2* targeting sequence from *Arabidopsis thaliana*, and the *TubA* 3′ untranslated region from *Oryza sativa*.

The backbone region of PV-ZMIR10871, located outside of the T-DNA, contains two origins of replication for maintenance of the plasmid vector in bacteria (ori V, ori-pBR322), a bacterial selectable marker gene (aadA), and a coding sequence for repressor of primer (ROP) protein for maintenance of plasmid vector copy number in Escherichia coli (E. coli). A description of the genetic elements and their prefixes (e.g., B-, P-, L-, I-, TS-, CS-, T-, and OR-) in PV-ZMIR10871 is provided in Table 3.

The *cry3Bb1* Coding Sequence and the Cry3Bb1 Protein

The *cry3Bb1* expression cassette encodes a 74.5 kDa Cry3Bb1 protein consisting of a single polypeptide of 653 amino acids (Figure 4). The *cry3Bb1* coding sequence is the codon optimized coding sequence from *Bacillus thuringiensis* that encodes the Cry3Bb1 protein (English et al., 2000). The presence of Cry3Bb1 protein provides protection from corn rootworm feeding.

The *cp4 epsps* Coding Sequence and the CP4 EPSPS Protein

The *cp4 epsps* expression cassette, encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Figure 5) (Padgette et al., 1996). The *cp4 epsps*

coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry et al., 2001; Padgette et al., 1996). The CP4 EPSPS protein is similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup herbicides, relative to endogenous plant EPSPS (Barry et al., 2001; Padgette et al., 1996). The presence of this protein renders the plant tolerant to Roundup.

DvSnf7^{p} Sequence

The DvSnf7^{p} sequence is the partial coding sequence of the *Snf7* gene from *Diabrotica virgifera virgifera* (Baum et al., 2007a; Baum et al., 2007b) encoding the SNF7 subunit of the ESCRT-III complex (Babst et al., 2002). The DvSnf7 suppression cassette contains two 240 bp DvSnf7^{p} sequences in an inverted orientation. There is an intervening sequence of 150 nucleotides between the two DvSnf7^{p} sequences (noted on Table 3 and Table 4). When the suppression cassette is transcribed, the RNA expressed forms a hairpin loop thereby allowing the formation of double stranded DvSnf7 RNA. The DvSnf7^{p} sequences in the suppression cassette produce a 240 bp dsRNA that upon transcription triggers the RNAi mechanism.

Regulatory Sequences

The *cry3Bb1* coding sequence in MON 87411 is under the regulation of the *pIIG* promoter, the chlorophyll a/b binding protein (CAB) leader, the *Ract1* intron, and the heat shock protein 17 (*Hsp17*) 3′ untranslated region. The *pIIG* promoter, which directs transcription in plant cells, is from the *pIIG* gene family encoding the physical impedance induced protein from *Zea mays* (Huang et al., 1998). The *CAB* leader is the 5' untranslated region from the chlorophyll a/b-binding (CAB) protein of *Triticum aestivum* and is involved in regulating gene expression (Lamppa et al., 1985). The *Ract1* intron is the intron from the *act1* gene from *Oryza sativa* (McElroy et al., 1990). The *Hsp17* 3′ non-translated region is the 3′ untranslated region from the heat shock protein, *Hsp17*, of *Triticum aestivum* (McElwain and Spiker, 1989) that directs polyadenylation of the mRNA.

The *cp4 epsps* coding sequence in MON 87411 is under the regulation of the *TubA* promoter, the *TubA* leader, the *TubA* intron, the *CTP2* targeting sequence, and the *TubA* 3′ untranslated region. The *TubA* promoter, which directs transcription in plant cells, is from the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al., 2000). The *TubA* intron is the intron from the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al., 2000). The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast in MON 87411 and is derived from CTP2 target sequence of the *Arabidopsis thaliana ShkG* gene (Herrmann, 1995; Klee et al., 1987). The *TubA* 3′ non-translated region is the 3′ untranslated region from the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al., 2000) that directs polyadenylation of mRNA.

The DvSnf7^{p} sequence in MON 87411 is under the regulation of the *e35S* promoter, the heat shock protein 70 (*Hsp70*) intron, and the *E9* 3′ untranslated region. The *e35S* promoter, which directs transcription in plant cells, contains the duplicated enhancer region (Kay et al., 1987) from the cauliflower mosaic virus (CaMV) *35S* RNA promoter (Odell et al., 1985). As demonstrated in this petition, the use of the CaMV 35S promoter containing the

duplicated enhancer region, derived from a designated plant pest, has not imparted plant pest characteristics to MON 87411. The *hsp70* intron is the first intron from the maize heat shock protein 70 gene (Brown and Santino, 1997; Rochester et al., 1986). The *E9* 3′ non-translated region is the 3′ untranslated region from the *rbcS* gene of *Pisum sativum* (pea) encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi et al., 1984) that directs polyadenylation of the mRNA.

T-DNA Borders

PV-ZMIR10871 contains Right Border and Left Border regions (Figure 6 and Table 3) that were derived from *Agrobacterium tumefaciens* plasmids. The border regions each contain a nick site that is the site of DNA exchange during transformation (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer of T-DNA into the maize genome. As demonstrated in this petition, the use of Right Border and Left Border regions derived from *Agrobacterium tumefaciens*, a designated plant pest, has not imparted plant pest characteristics to MON 87411.

Genetic Elements Outside of the T-DNA Borders

Genetic elements that exist outside of the T-DNA borders are those that are essential for the maintenance or selection of PV-ZMIR10871 in bacteria. The origin of replication *ori V* is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host plasmid RK2 (Stalker et al., 1981). The origin of replication *ori-pBR322* is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe, 1979). Coding sequence *rop* is the coding sequence of the repressor of primer (ROP) protein and is necessary for the maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989). The selectable marker aadA is a bacterial promoter and coding sequence for an enzyme from transposon Tn*7* that confers spectinomycin and streptomycin resistance (Fling et al., 1985) in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the maize genome. The absence of the backbone and other unintended plasmid sequence in MON 87411 has been confirmed by sequencing and bioinformatic analyses (see Section A3(d)(ii)).

Table 3 Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al.*, 1983)
Intervening Sequence	443-485	Sequence used in DNA cloning
T[2]-*E9*	486-1118	3′ UTR of the *rbcS* gene family from *Pisum sativum* (pea) encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi *et al.*, 1984) that directs polyadenylation of the mRNA
Intervening Sequence	1119-1147	Sequence used in DNA cloning
DvSnf7[p]	1148-1387	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum *et al.*, 2007a; Baum *et al.*, 2007b) encoding the SNF7 subunit of the ESCRT-III complex (Babst *et al.*, 2002) that forms part of the suppression cassette
Intervening Sequence	1388-1537	Sequence used in DNA cloning
DvSnf7[p]	1538-1777	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum *et al.*, 2007a; Baum *et al.*, 2007b) encoding the SNF7 subunit of the ESCRT-III complex (Babst *et al.*, 2002) that forms part of the suppression cassette
Intervening Sequence	1778-1813	Sequence used in DNA cloning
I[3]-*Hsp70*	1814-2617	Intron and flanking exon sequence of the *hsp70* gene from *Zea mays* (maize) encoding the heat shock protein 70 (HSP70) (Rochester *et al.*, 1986) that is involved in regulating gene expression (Brown and Santino, 1997)
P[4]-*e35S*	2618-3238	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell *et al.*, 1985) containing the duplicated enhancer region (Kay *et al.*, 1987) that directs transcription in plant cells
Intervening Sequence	3239-3264	Sequence used in DNA cloning

Table 3 (continued) Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
P-*pIIG*	3265-4213	Promoter sequence of the *pIIG* gene encoding the physical impedance induced protein from *Zea mays* (Huang *et al.*, 1998) (maize) that directs transcription in plant cells
Intervening Sequence	4214-4219	Sequence used in DNA cloning
L[5]-*Cab*	4220-4280	5' UTR leader sequence from chlorophyll a/b-binding (CAB) protein of *Triticum aestivum* (wheat) that is involved in regulating gene expression (Lamppa *et al.*, 1985)
Intervening Sequence	4281-4296	Sequence used in DNA cloning
I-*Ract1*	4297-4776	Intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 protein is involved in regulating gene expression (McElroy *et al.*, 1990)
Intervening Sequence	4777-4785	Sequence used in DNA cloning
CS[6]-*cry3Bb1*	4786-6747	Codon optimized coding sequence from Cry3Bb1 protein of *Bacillus thuringiensis* that provides insect resistance (English *et al.*, 2000)
Intervening Sequence	6748-6766	Sequence used in DNA cloning
T-*Hsp17*	6767-6976	3' UTR sequence from a heat shock protein, Hsp17, of *Triticum aestivum* (wheat) (McElwain and Spiker, 1989) that directs polyadenylation of the mRNA
Intervening Sequence	6977-7024	Sequence used in DNA cloning
P-*TubA*	7025-9205	Promoter, 5′UTR leader and intron sequences of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon *et al.*, 2000) that directs transcription in plant cells
Intervening Sequence	9206-9209	Sequence used in DNA cloning

Table 3 (continued) Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
TS[7]-*CTP2*	9210-9437	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee *et al.*, 1987)
CS-*cp4 epsps*	9438-10805	Codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding the native CP4 EPSPS protein that provides herbicide tolerance (Barry *et al.*, 2001; Padgette *et al.*, 1996)
Intervening Sequence	10806-10812	Sequence used in DNA cloning
T-*TubA*	10813-11394	3' UTR sequence of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon *et al.*, 2000) that directs polyadenylation of mRNA
Intervening Sequence	11395-11412	Sequence used in DNA cloning
B-Right Border Region	11413-11743	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker *et al.*, 1982; Zambryski *et al.*, 1982)
Vector Backbone		
Intervening Sequence	11744-11879	Sequence used in DNA cloning
aadA	11880-12768	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from the transposon Tn*7* (Fling *et al.*, 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	12769-13298	Sequence used in DNA cloning
OR[8]-*ori-pBR322*	13299-13887	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	13888-14314	Sequence used in DNA cloning
CS-*rop*	14315-14506	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	14507-16014	Sequence used in DNA cloning

Table 3 (continued) Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
OR-*ori V*	16015-16411	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker *et al.*, 1981)
Intervening Sequence	16412-16497	Sequence used in DNA cloning

[1] B, Border
[2] T, Transcription Termination Sequence
[3] I, Intron
[4] P, Promoter
[5] L, Leader
[6] CS, Coding Sequence
[7] TS, Targeting Sequence
[8]OR, Origin of Replication
[p] Superscript in DvSnf7 indicates the partial sequence. Within the DvSnf7 cassette, bases 1148-1387 are reverse complement to bases 1538-1777.

```
  1 MANPNNRSEH 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 4 Deduced Amino Acid Sequence of the Cry3Bb1 Protein

The amino acid sequence of the Cry3Bb1 protein was deduced from the full-length coding nucleotide sequence present in PV-ZMIR10871.

```
  1 MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG LKKSGMTLIG
 61 SELRPLKVMS SVSTACMLHG ASSRPATARK SSGLSGTVRI PGDKSISHRS FMFGGLASGE
121 TRITGLLEGE DVINTGKAMQ AMGARIRKEG DTWIIDGVGN GGLLAPEAPL DFGNAATGCR
181 LTMGLVGVYD FDSTFIGDAS LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP
241 ITYRVPMASA QVKSAVLLAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
301 TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR TGLILTLQEM
361 GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM IDEYPILAVA AAFAEGATVM
421 NGLEELRVKE SDRLSAVANG LKLNGVDCDE GETSLVVRGR PDGKGLGNAS GAAVATHLDH
481 RIAMSFLVMG LVSENPVTVD DATMIATSFP EFMDLMAGLG AKIELSDTKA A
```

Figure 5 Deduced Amino Acid Sequence of the CTP2 Targeting Sequence and CP4 EPSPS Protein

The transit peptide CTP2 for the CP4 EPSPS protein is underlined. Accumulation of the CP4 EPSPS protein is targeted to the chloroplasts using cleavable CTP2, the transit peptide of the *Arabidopsis thaliana* EPSPS protein. The amino acid sequence of the CP4 EPSPS protein was deduced from the full-length coding nucleotide sequence present in PV-ZMIR10871.

A3(c)(ii) Detailed map of the location and orientation of all genetic elements

Plasmid map with locations of genetic elements are shown in Figure 6.

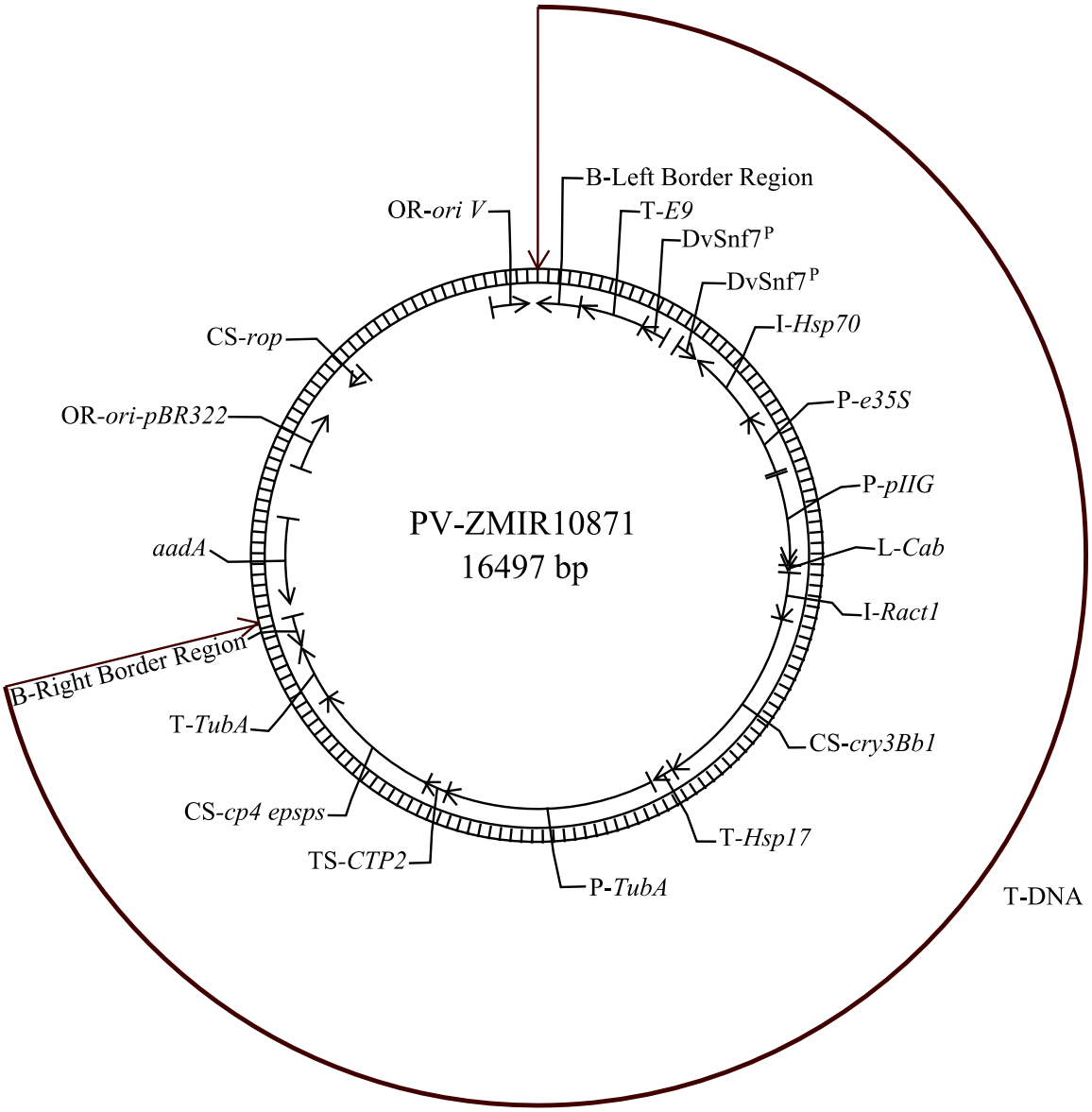


Figure 6 Circular Map of PV- ZMIR10871

A circular map of the plasmid vector PV-ZMIR10871 used to develop MON 87411 is shown. PV-ZMIR10871 contains a single T-DNA. Genetic elements are shown on the exterior of the map. [P] Superscript in DvSnf7 indicates partial sequence.

A3(d)(i) Molecular characterisation including identification of GM elements

Characterization of the DNA insert in MON 87411 was conducted using a combination of sequencing, PCR and bioinformatics. The results of this characterization demonstrate that MON 87411 contains one copy of the intended transfer DNA (T-DNA) containing the DvSnf7 suppression cassette and the *cry3Bb1* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions are based on several lines of evidence:

- Molecular characterization of MON 87411 by Next Generation Sequencing and Junction Sequence Analysis (NGS/JSA) demonstrated that MON 87411 contained a single DNA insert. These whole-genome sequence analyses provided a comprehensive assessment of MON 87411 to determine the presence of sequences derived from PV-ZMIR10871 (Kovalic *et al.*, 2012; DuBose *et al.*, 2013), demonstrated that MON 87411 contained a single DNA insert.
- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) of MON 87411 was used to determine the complete sequence of the single DNA insert from PV-ZMIR10871, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the sequence and organization of the DNA is identical to the corresponding region in the PV-ZMIR10871 T-DNA. The sequencing analysis, along with the NGS/JSA result showing that MON 87411 contains only a single DNA insert with no unintended fragments, also confirms that no vector backbone or other unintended plasmid sequences are present in MON 87411. Furthermore, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 87411 to the sequence of the insertion site in conventional maize. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 87411 upon DNA integration.
- Generational stability analysis by NGS/JSA demonstrated that the single PV-ZMIR10871 T-DNA insert in MON 87411 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 87411.
- Segregation analysis corroborates the insert stability demonstrated by NGS/JSA and independently establishes the nature of the T-DNA as a single chromosomal locus.

Taken together, the characterization of the genetic modification in MON 87411 demonstrates that a single copy of the intended T-DNA was stably integrated at a single locus of the maize genome and that no plasmid backbone sequences are present in MON 87411.

A schematic representation of the NGS/JSA methodology and the basis of the characterization using NGS/JSA and PCR sequencing are illustrated in Figure 7 below.

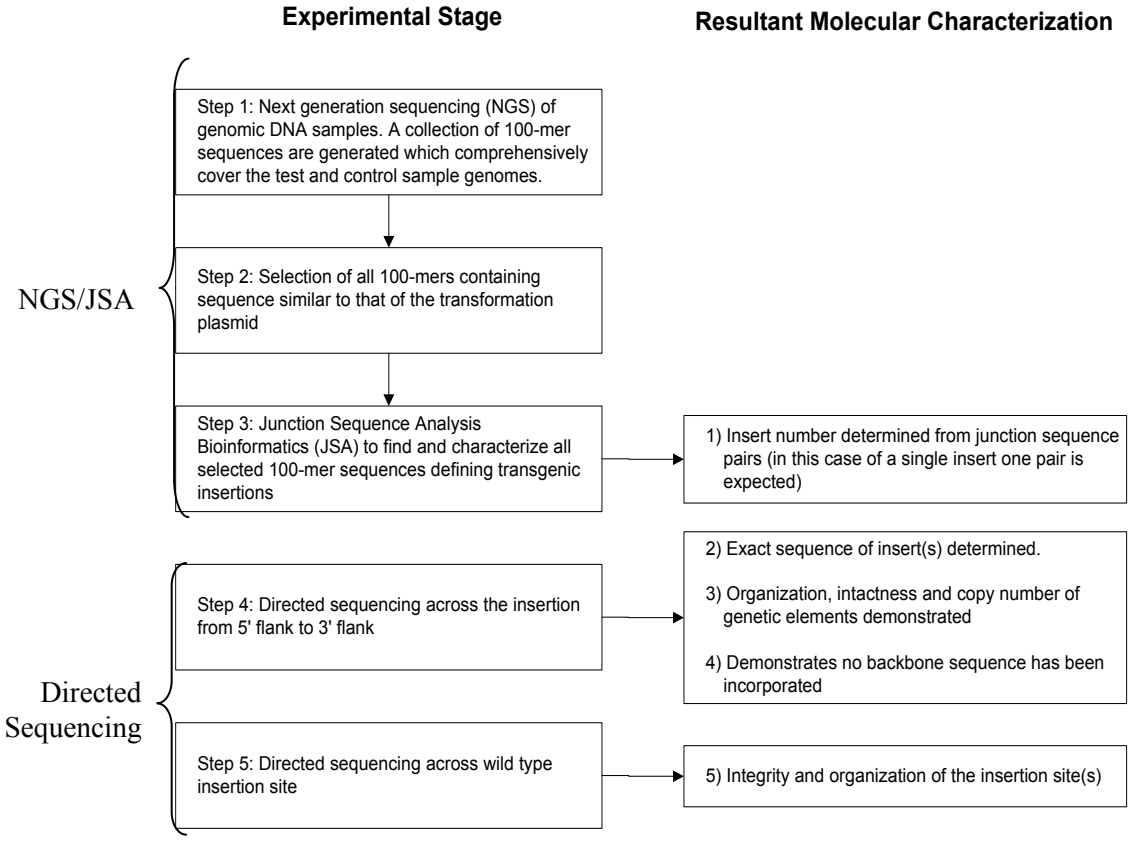


Figure 7 Molecular Characterization using Sequencing and Bioinformatics

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).

The NGS/JSA method characterized the genomic DNA from MON 87411 and the conventional control using short (~100 bp) randomly distributed sequence fragments (sequencing reads) generated in sufficient number to ensure comprehensive coverage of the sample genomes. Bioinformatics analysis was then used to select sequencing reads that contained sequences similar to the transformation plasmid, and these were analysed to determine the number of DNA inserts. NGS/JSA was run on all MON 87411 samples and the conventional controls; results of NGS/JSA are shown in Section A3 (d)(ii) below.

The number of DNA inserts was determined by analyzing sequences for novel junctions. The junctions of the DNA insert and the flanking DNA are unique for each insertion; an example is shown in Figure 8 below (Kovalic *et al.*, 2012). Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions.

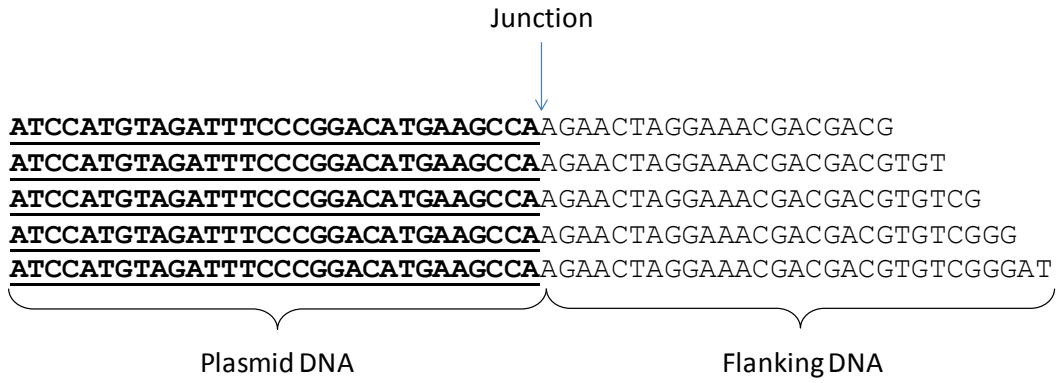


Figure 8 Junctions and Junction Sequences

Depicted above are five example junction sequences formatted and labeled to indicate the plasmid/flanking DNA portions of the sequences and with the junction point indicated (plasmid DNA is shown in bold, underlined text and flank DNA is shown in plain text). Junctions are detected by examining the NGS data for sequences having portions of plasmid sequences that span less than the full read. Detected junctions are typically characteristic of plasmid insertions in the genome.

Each insertion will produce two unique junction sequence classes characteristic of the genomic locus, one at the 5' end of the insert (illustrated in see Figure 9 below, and named junction sequence class A, or JSC-A, in this case) and one at the 3' end of the insert, named junction sequence class B, or JSC-B, in this case (Kovalic *et al.*, 2012).

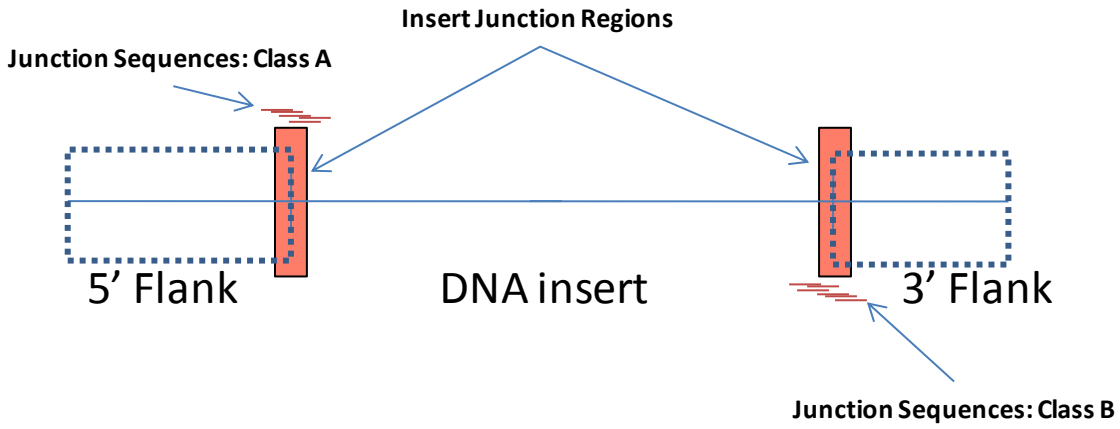


Figure 9 Two Unique Junction Sequence Classes are Produced by the Insertion of a Single Plasmid Region

A schematic representation of a single DNA insertion within the genome showing the inserted DNA, the 5' and 3' flanks (depicted as areas bounded by dotted lines), and the two distinct regions spanning the junctions between inserted DNA and flanking DNA (shaded boxes). The group of ~100-mer sequences in which each read contains sequences from both the DNA insert and the adjacent flanking DNA at a given junction is called a Junction Sequence Class. In this example, two distinct junction sequence classes (in this case: Class A at the 5' end and Class B at the 3' end) are represented.

By evaluating the number of unique junction classes detected, the number of insertion sites of the plasmid sequence can be determined. For a single insert two junction sequence classes are expected, one each originating from either end of the insert, both containing portions of plasmid DNA insert and flanking sequence. In the case of MON 87411, two unique junction sequence classes, both containing portions of T-DNA and flanking sequence, were detected indicating MON 87411 contains a single DNA insert (results are described in Section A3(d)(ii)). The identity of the DNA insert (i.e., T-DNA or backbone) is determined by direct sequencing described below.

The NGS/JSA strategy to determine insert number of the integrated plasmid DNA was designed to ensure that all transgenic segments would be identified. The depth of coverage (the average number of times each base of the genome is independently sequenced) was ≥75× for each sample genome. It has previously been demonstrated that ≥75× coverage of the soybean genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic *et al.*, 2012) and similarly ≥75× coverage provides comprehensive coverage of the maize genome (Clarke and Carbon, 1976). The level of sensitivity of this method was demonstrated by detection of a positive control spiked at $1/10^{th}$ copy-per-genome equivalent.

Directed sequencing (locus-specific PCR and DNA sequencing analyses, Figure 7, step 4) complements the NGS/JSA analyses. As indicated above, NGS/JSA results determined that MON 87411 contains a single DNA insertion site. Sequencing of the insert and flanking

genomic DNA determined the complete sequence of the insert and flanks; it determined that the sequence and organization of the DNA insert is identical to those in the T-DNA of PV-ZMIR10871, and that each genetic element (except for the border regions) in the insert is intact, and also that no vector backbone, or other unintended plasmid sequences were inserted in MON 87411. Furthermore, the genomic organization at the insertion site was assessed by comparing the insert and MON 87411 flanking sequence to the sequence of the insertion site in conventional maize. This assessment indicated that the integration site in the MON 87411 genome included a 118 bp deletion of genomic DNA but is otherwise identical to the native sequence. Results are described in Section A3(d)(ii).

The stability of the T-DNA present in MON 87411 across multiple generations was evaluated by NGS/JSA analyses. Genomic DNA from five generations of MON 87411 (Figure 10) was assayed for all unique junction sequence classes as described above. This information was used to determine the number and identity of insertion sites. For a single insert, two junction sequence classes are expected; each one originates from either end of the insert, both containing portions of DNA insert and flanking sequence. In the case of MON 87411, two identical junction sequence classes were detected in all the generations tested, confirming that the single insert is stably inherited over multiple generations.

Segregation analysis of the T-DNA was conducted to determine the inheritance and stability of the insert in MON 87411. Results from this analysis demonstrate inheritance according to Mendelian principles and the stability of the insert is as expected across multiple generations, (Table 7, Table 8, Figure 18). The segregation analysis corroborates the insert stability demonstrated by NGS/JSA and independently establishes the genetic behavior of the T-DNA as a single chromosomal locus.

The results of these analyses of MON 87411 demonstrate that a single copy of the intended T-DNA derived from PV-ZMIR10871 was inserted at a single locus of the MON 87411 genome, that the sequence and organization of the T-DNA insert is identical to the corresponding region in PV-ZMIR10871 and that no additional genetic elements, including backbone sequences, were detected in MON 87411. Generational stability analysis demonstrated that the single insert in MON 87411 was maintained through five generations of the breeding history, thereby confirming the stability of T-DNA in MON 87411. In addition, results from segregation analyses confirmed the genetic behavior of the T-DNA as a single chromosomal locus.

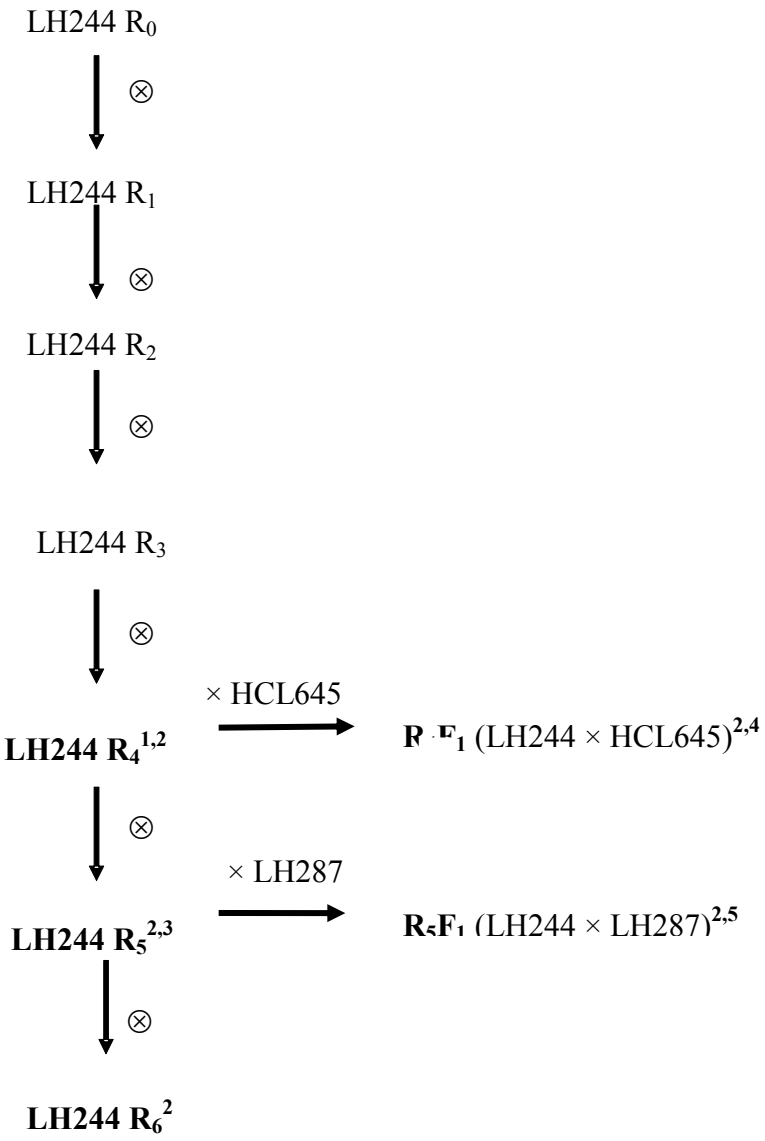


Figure 10 Breeding History of MON 87411

R_0 corresponds to the transformed plant, $F_{\#}$ is the filial generation, $R_{\#}$ is the regenerant and subsequent generations, ⊗ designates self-pollination.

[1]Generation used for full molecular characterization.

[2] Generations used to confirm insert stability.

[3] Generation used for commercial development of MON 87411.

[4] Generation used for compositional analysis and RNA expression studies.

[5] Generation used for agronomic/phenotypic studies

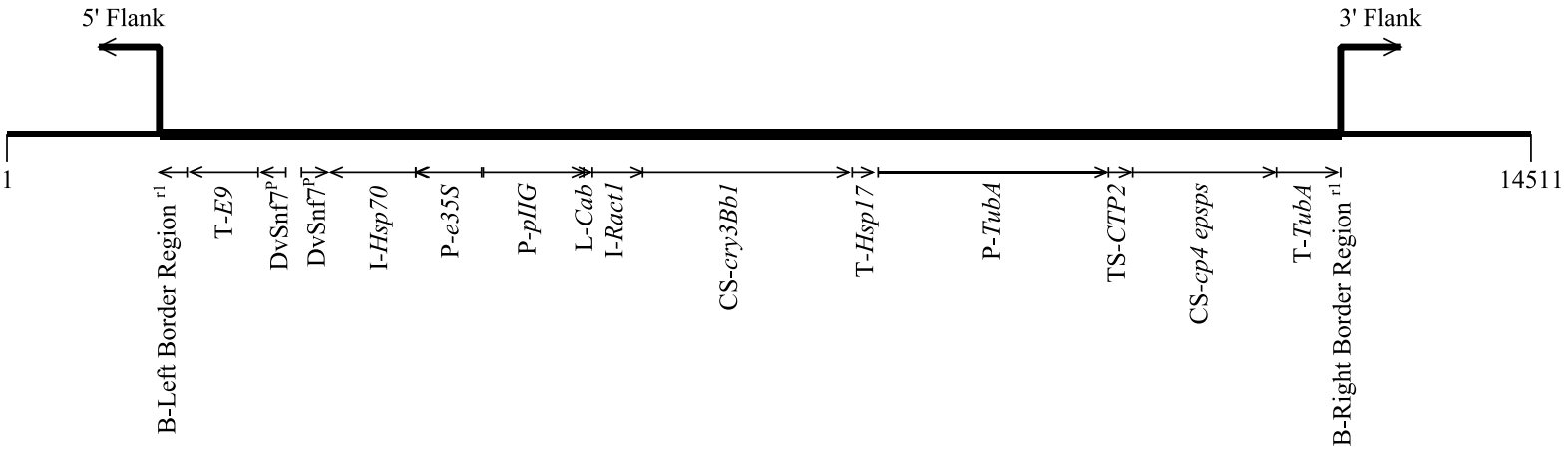


Figure 11 Schematic Representation of the Insert and Flanking Sequences in MON 87411

Linear map showing DNA derived from the T-DNA of PV-ZMIR10871 integrated into MON 87411. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking sequence. Identified on the map are genetic elements within the insert. This schematic diagram is drawn to scale; the exact coordinates of every element is shown in Table 4.

r1 Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.

P Superscript in DvSnf7 indicates the partial sequence.

Table 4 Summary of Genetic Elements in MON 87411

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
5′ Flank	1-1460	Sequence flanking the 5′ end of the insert
B[3]-Left Border Region[r1]	1461-1723	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al.*, 1983)
Intervening Sequence	1724-1766	Sequence used in DNA cloning
T[4]-*E9*	1767-2399	3' UTR sequence from *Pisum sativum rbcS* gene family encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi *et al.*, 1984) that directs polyadenylation of the mRNA
Intervening Sequence	2400-2428	Sequence used in DNA cloning
DvSnf7[P]	2429-2668	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum *et al.*, 2007a; Baum *et al.*, 2007b) encoding the Snf7 subunit of the ESCRT-III complex (Babst *et al.*, 2002) that forms part of the suppression cassette
Intervening Sequence	2669-2818	Sequence used in DNA cloning
DvSnf7[P]	2819-3058	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum *et al.*, 2007a; Baum *et al.*, 2007b) encoding the Snf7 subunit of the ESCRT-III complex (Babst *et al.*, 2002) that forms part of the suppression cassette
Intervening Sequence	3059-3094	Sequence used in DNA cloning
I[5]-*Hsp70*	3095-3898	Intron and flanking exon sequence of the *hsp70* gene from *Zea mays* (maize) encoding the heat shock protein 70 (HSP70) (Rochester *et al.*, 1986)is involved in regulating gene expression (Brown and Santino, 1997)
P[6]-*e35S*	3899-4519	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell *et al.*, 1985) containing the duplicated enhancer region (Kay *et al.*, 1987) that directs transcription in plant cells
Intervening Sequence	4520-4545	Sequence used in DNA cloning
P-*pIIG*	4546-5494	Promoter sequence from the physical impedance induced protein of *Zea mays* (maize) (Huang *et al.*, 1998) that directs transcription in plant cells

Table 4 (continued) Summary of Genetic Elements in MON 87411

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Intervening Sequence	5495-5500	Sequence used in DNA cloning
L[7]-*Cab*	5501-5561	5' UTR leader sequence from chlorophyll a/b-binding (CAB) protein of *Triticum aestivum* (wheat) that is involved in regulating gene expression (Lamppa *et al.*, 1985)
Intervening Sequence	5562-5577	Sequence used in DNA cloning
I-*Ract1*	5578-6057	Intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 protein is involved in regulating gene expression (McElroy *et al.*, 1990)
Intervening Sequence	6058-6066	Sequence used in DNA cloning
CS[8]-*cry3Bb1*	6067-8028	Codon optimized coding sequence for Cry3Bb1 protein of *Bacillus thuringiensis* that provides insect resistance (English *et al.*, 2000)
Intervening Sequence	8029-8047	Sequence used in DNA cloning
T-*Hsp17*	8048-8257	3' UTR sequence from a heat shock protein, HSP17, of *Triticum aestivum* (wheat) (McElwain and Spiker, 1989) that directs polyadenylation of the mRNA
Intervening Sequence	8258-8305	Sequence used in DNA cloning
P-*TubA*	8306-10486	Promoter, 5′ UTR leader and intron sequences of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon *et al.*, 2000) that directs transcription in plant cells
Intervening Sequence	10487-10490	Sequence used in DNA cloning
TS[9]-*CTP2*	10491-10718	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee *et al.*, 1987)
CS-*cp4 epsps*	10719-12086	Coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding the native CP4 EPSPS protein that provides herbicide tolerance (Barry *et al.*, 2001; Padgette *et al.*, 1996)
Intervening Sequence	12087-12093	Sequence used in DNA cloning
T-*TubA*	12094-12675	3' UTR sequence of the *OsTubA* gene from *Oryza sativa* (rice) encoding α-tubulin (Jeon *et al.*, 2000) that directs polyadenylation of mRNA

Table 4 (continued) Summary of Genetic Elements in MON 87411

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Intervening Sequence	12676-12693	Sequence used in DNA cloning
B-Right Border Region[r1]	12694-12708	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker *et al.*, 1982; Zambryski *et al.*, 1982)
3′ Flank	12709-14511	Sequence flanking the 3′ end of the insert

[1] Although flanking sequences and intervening sequence are not functional genetic elements, they comprise a portion of the sequence.
[2] Numbering refers to the sequence of the insert in MON 87411 and adjacent DNA.
[3] B, Border
[4] T, Transcription Termination Sequence
[5] I, Intron
[6] P, Promoter
[7] L, Leader
[8] CS, Coding Sequence
[9] TS, Targeting Sequence
[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871
[P] Superscript in DvSnf7 indicates the partial sequence. Within the DvSnf7 cassette, bases 2429-2668 are reverse complement to bases 2819-3058.

Please also refer to Carleton *et al.*, 2013 (MSL0025314).

A3(d)(ii) Determination of number of insertion sites, and copy number

Determining the Number of DNA inserts in MON 87411

The number of insertion sites of PV-ZMIR10871 DNA in the MON 87411 was assessed by performing NGS/JSA on MON 87411 genomic DNA. A single genomic DNA insertion is expected to produce two junction sequence classes and any additional integration sites would produce additional junction sequence classes. A plasmid map of PV-ZMIR10871 is shown in Figure 6 . A schematic representation of the insert and flanking sequences in MON 87411 is shown in Figure 11. The generations studied are depicted in the breeding history diagram shown in Figure 10. The NGS conducted for all samples and its adequate depth of coverage in each case is summarized in below Section of A3(d)(ii) and Table 5. The sensitivity of the method is demonstrated below with data shown in Table 6. The JSA analysis of the R_4 generation is shown below with data presented in Figure 12; the other generations that were used in the generational stability analysis are shown in Figure 10 with the results of JSA analysis described in Section A3(f).

Next Generation Sequencing (NGS) for MON 87411 and Conventional Control Genomic DNA

Genomic DNA from five generations of MON 87411 and the conventional controls (inbred LH244 and hybrids NL6169 and MPA640B) were isolated and prepared for sequencing according to the manufacturer's protocol (Illumina, TruSeq library protocol. . These genomic DNA libraries were used to generate short (~100 bp) randomly distributed sequence fragments (sequencing reads) in sufficient numbers to ensure comprehensive coverage of the maize genome (see Figure 8, Step 1).

To confirm sufficient sequence coverage in all generations of MON 87411 and the conventional controls, the 100-mer sequence reads from all samples were analyzed 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 gene (Pyruvate decarboxylase (*pdc3*), GenBank accession.version: AF370006.2). The analysis showed that *pdc3* was covered by 100-mers at >107× for each sample (Table 5). It has previously been demonstrated that ≥75× coverage of the soybean genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic *et al.*, 2012) and similarly ≥75× coverage provides comprehensive coverage of the maize genome (Clarke and Carbon, 1976). A summary of NGS and effective depth of coverage are shown in Table 5.

In order to confirm the method's ability to detect any sequences derived from the PV-ZMIR10871 transformation plasmid, a sample of conventional control maize DNA spiked with PV-ZMIR10871 DNA at 1 and $1/10^{th}$ genome equivalent was analyzed by NGS and bioinformatics. At 1 genome equivalent, 100% nucleotide identity was observed over 100% of PV-ZMIR10871 (Table 6). This result demonstrates that all nucleotides of the transformation plasmid are observed by the sequencing and bioinformatic assessments performed. Also,

observed coverage was adequate (Clarke and Carbon, 1976) at a level of at most 1/10th genomic equivalent (99.64% coverage at 100% identity for the 1/10th genome equivalent spiked control sample, Table 6) and, hence, a detection level of at least 1/10th genome equivalent was achieved for the plasmid DNA sequence assessment.

Table 5 Sequencing (NGS) Conducted for MON 87411 and Control Genomic DNA

Sample	Total Nucleotides (Gb)	Effective Median Depth of Coverage (x-fold)
LH244	346.9	126x
LH244 × HCL645	309.5	107x
LH244 × LH287	342.3	118x
R_4	334.9	125x
R_4F_1	338.9	113x
R_5F_1	346.6	126x
R_5	352.3	140x
R_6	363.9	134x

For each sample the raw data produced are presented in terms of total nucleotide number. Effective depth of coverage is determined by mapping and alignment of all raw data to a well known single copy locus (*pdc3*: pyruvate decarboxylase) within the maize genome. The median effective depths of coverage are shown for all samples.

Table 6 Summary of NGS Data for the Conventional Control DNA Sample Spiked with PV-ZMIR10871 DNA

	1/10th copy Spike	1 copy Spike
Extent of coverage[1] of PV-ZMIR10871	99.64%	100%
Percent identity of coverage[2] of PV-ZMIR10871	100%	100%

[1] Extent of coverage is calculated as the percent of all PV-ZMIR10871 bases observed in the sequencing of the spike-in samples:

$$\text{extent of coverage} = \frac{\text{number of spike in bases detected}}{\text{total length (bp) of spike in plasmid}} \times 100$$

[2] Percent identity of coverage is calculated as the percent of all PV-ZMIR10871 bases observed in the sequencing of the spike-in samples:

$$\text{Percent identity of coverage} = \frac{\text{number of identical bases (spike in vs. plasmid sequence) detected}}{\text{total length (bp) of spike in plasmid detected}} \times 100$$

Characterization of insert number in MON 87411 using Bioinformatic Analysis

The number of insertion sites of DNA from PV-ZMIR10871 in the MON 87411 was assessed by performing NGS/JSA on MON 87411 genomic DNA using the R_4 generation (Figure 10). A single genomic DNA insertion is expected to produce two junction sequence classes and any additional integration sites would produce additional junction sequence classes.

Selection of Sequence Reads Containing Sequence of the PV-ZMIR10871

PV-ZMIR10871 was transformed into the parental line LH244 to produce MON 87411. Consequently, any DNA inserted into MON 87411 will consist of sequences that are similar to the PV-ZMIR10871 DNA sequence. To fully characterize the DNA from PV-ZMIR10871 inserted in MON 87411, it is sufficient to completely analyze only the sequence reads that have similarity to the transformation plasmid (Figure 7, Step 2). In order to analyze the sequence data for insert number, all sequences that have significant sequence similarity to PV-ZMIR10871 were selected from the full sequencing datasets (Kovalic et al., 2012). Due to the depth of sequence coverage demonstrated with this methodology, on average, any area of the genome will be covered by more than 107 of the 100-mer sequences; this ensures that sequences from PV-ZMIR10871 inserted into the genome will be detected by the analysis.

Using established criteria, reads similar to the transformation plasmid were selected from MON 87411 and the conventional control sequence datasets and were then used as input data for bioinformatic junction sequence analysis.

Determination of the Insert Number

The NGS/JSA method described above used the entire PV-ZMIR10871 plasmid as a query to determine the DNA insertion site number. Any DNA inserts, regardless of whether the sequence was from backbone or T-DNA, can be detected by junction sequences. Therefore, unlike the traditional Southern blot analysis that separately hybridizes T-DNA or backbone probes, in NGA/JSA the determination of the T-DNA insert number and of the absence of backbone or unintended sequences are simply represented by the determination of the overall insert number in the genome followed by determination of the exact identity of any DNA insert using directed sequencing and sequence analysis.

By evaluating the number of unique junction classes, the number of DNA insertion sites can be determined (Figure 7, Step 3). For a single insert, at a single genomic locus, a single pair of junction sequences classes, each one originating from either end of the insert, is expected. If MON 87411 contains a single T-DNA insert two junction sequence classes (JSCs) each containing portions of T-DNA sequence and flanking sequence will be detected.

To determine the insert number in MON 87411, the selected sequence reads described above were analyzed using JSA (Kovalic et al., 2012). JSA uses bioinformatic analysis to find and classify partially matched reads characteristic of the ends of insertions. The number of resultant unique JSCs were determined by this analysis and are shown in **Table 7** below.

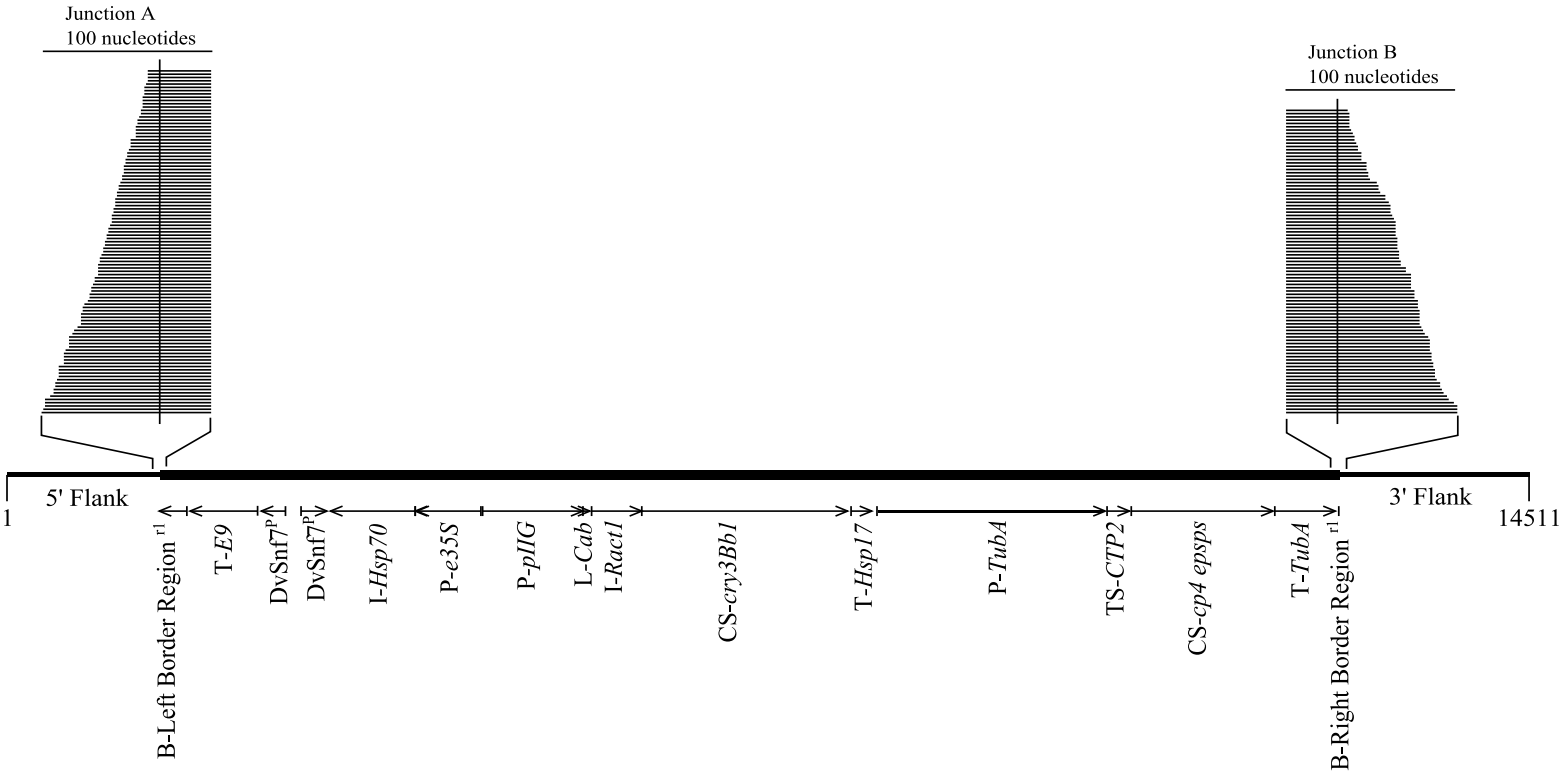
Table 7 Unique Junction Sequence Class Results

Sample	JSCs Detected
MON 87411	2
LH244	0

Detailed sequence information of the junction sequences detected by JSA is shown in Figure 12 (Panel B). The location and orientation of the junction sequences relative to the T-DNA insert determined for MON 87411 is shown in Figure 12 (Panel A). As shown in the figure, there are two junction sequence classes identified in MON 87411. Junction Sequence Class A and Class B (JSC-A and JSC-B) both contain the T-DNA border sequence joined to flanking sequence, indicating that they represent the sequences at the junctions of the intended T-DNA insert and flanking sequence.

The presence of two, and only two, junction sequence classes (joining T-DNA border and flanking sequences) indicate this single pair of JSCs arises from the insertion of the intended PV-ZMIR10871 T-DNA at a single locus in the genome of MON 87411. JSC-A represents the junction of the T-DNA Left Border sequence to the 5' flank and JSC-B represents the junction of the T-DNA Right Border sequence to the 3' flank. As shown by exact and complete alignment of the JSCs to the full flank/insert sequence (shown in Figure 12, Panel B) both of these JSCs originate from the same locus of the MON 87411 genome and are linked by contiguous, known and expected DNA that makes up the single insert.

Based on this comprehensive NGS/JSA study it is concluded that MON 87411 contains one DNA inserted into a single locus, as shown in Figure 11. The identity of the DNA insert was determined by the sequencing and analysis of overlapping PCR products from this locus as described below in Section A3(d)(iii), which showed that the DNA insert only contained the T-DNA elements from the plasmid and the backbone sequences were not present. These results demonstrated that the DNA insert only contained the T-DNA elements from the plasmid and no backbone sequences from the plasmid existed in MON 87411.



Panel A.

Figure 12 Junction Sequences Detected by NGS/JSA

Panel A: Linear map of MON 87411 illustrating the relationship of the detected junction sequences to the insert locus. The individual junction sequences detected by JSA are illustrated as stacked bars; each detected junction sequence read is shown trimmed to include only 30 bases of plasmid sequence. The scale of the identified junction sequences relative to the insert map is depicted by the braces.
[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.
[P] Superscript in DvSnf7 indicates the partial sequence.

```
JSC-A Alignment:
JSC_A:             1   AATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCATT   60
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 1554    AATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCATT   1495

JSC_A:            61   GCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCCT   120
                       |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Directed Seq.: 1494    GCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCCT   1435

JSC_A:           121   TTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAACC    164
                       |||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 1434    TTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAACC    1391

JSC-B Alignment:
JSC-B:             1   GGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACACCA   60
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 12616   GGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACACCA   12675

JSC-B:            61   TCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGTATT   120
                       |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Directed Seq.: 12676   TCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGTATT   12735

JSC-B:           121   AATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT    163
                       |||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 12736   AATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT    12778
```

Panel B.

Figure 12 (continued) Junction Sequences Detected by NGS/JSA

Panel B: Full consensus sequence for junction sequence Classes A and B (JSC-A and JSC-B) showing exact alignment to the independently determined *in planta* sequence at the insert locus (labeled "Directed Seq."). The numbers flanking the sequence text represent the base pair numbering of the JSA consensus sequence or the insert sequence, respectively. Double underlined text indicates plasmid DNA sequence, single underlined text indicates plant genome sequence, and the carat character "^" indicates the junction point between the MON 87411 insert and the flank.

A3(d)(iii) Full DNA sequence, including junction regions

Organisation and Sequence of the Insert and Adjacent Flanking DNA in MON 87411

The organization of the elements within the DNA insert and the adjacent genomic DNA was assessed using directed DNA sequence analysis (refer to Figure 7, Step 4). PCR primers were designed to amplify eight overlapping regions of the MON 87411 genomic DNA that span the entire length of the insert (Figure 13). The amplified PCR products were subjected to DNA sequencing analyses. The results of this analysis confirm that the MON 87411 insert is 11,248 bp and that each genetic element in the insert is intact, with the exception of the Right and Left border regions. The border regions both contain small terminal deletions with the remainder of the inserted border regions being identical to the sequence in PV-ZMIR10871. The sequence and organization of the insert was also shown to be identical to the corresponding T-DNA of PV-ZMIR10871, confirming that a single copy of T-DNA was inserted as intended. This analysis also shows that only T-DNA elements (described in Table 4) were present. Moreover, the result, together with the conclusion of single DNA insert detected by NGS/JSA, demonstrated that no PV-ZMIR10871 backbone sequences were present in MON 87411.

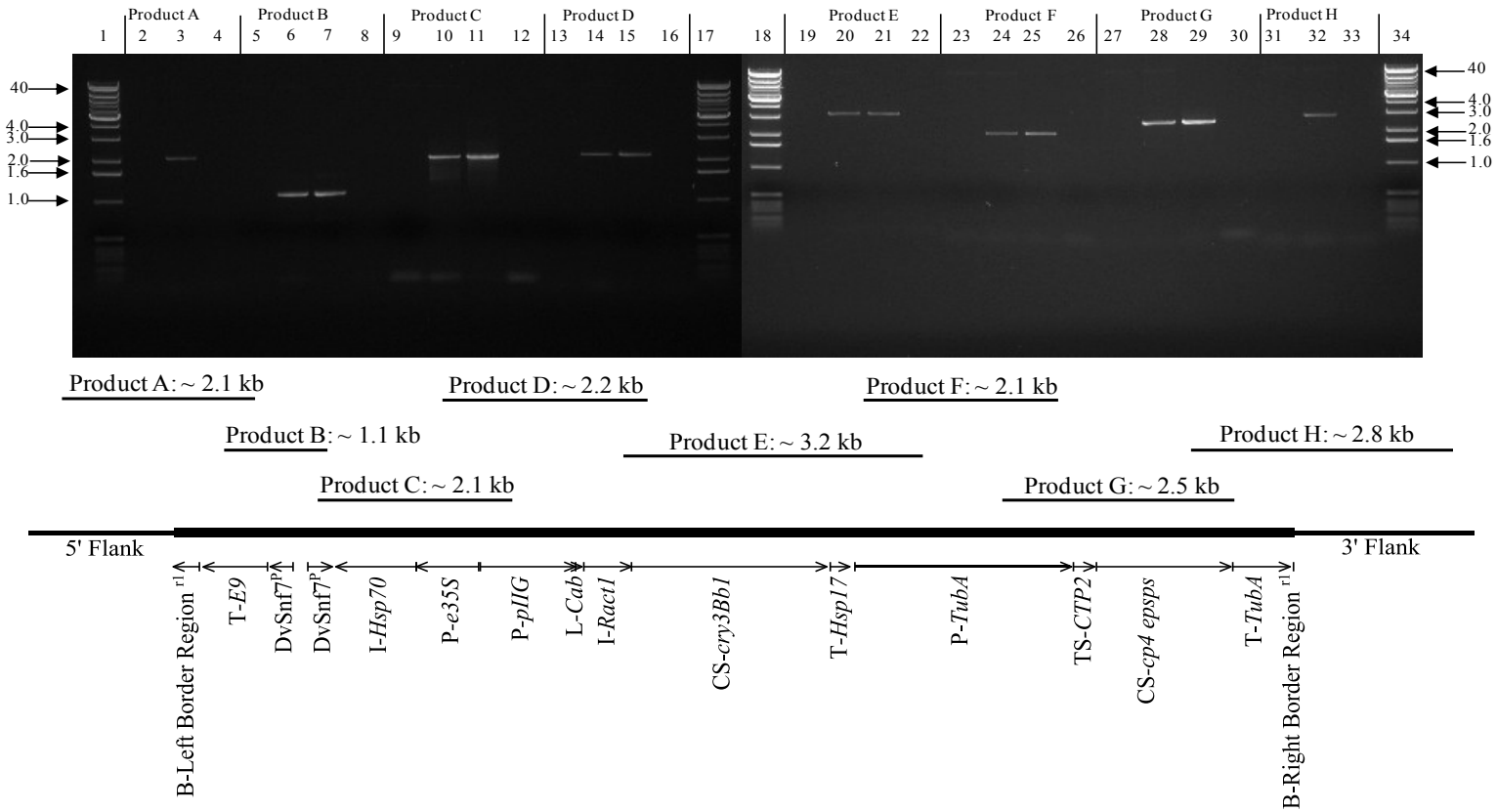


Figure 13 Analysis of Overlapping PCR Products Across the MON 87411 Insert

PCR was performed on both conventional control genomic DNA and genomic DNA of the R_4 generation of MON 87411 using eight pairs of primers to generate overlapping PCR fragments from MON 87411 for sequencing analysis. To verify the production of PCR products, 5 µl of each of the PCR reactions was loaded on the gel, except where noted below. The expected product size for each amplicon is provided in the illustration.

[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.

[P] Superscript in DvSnf7 indicates the partial sequence.

Lane designations are as follows:

Lane	Sample	Lane	Sample
1	1 kb DNA Extension Ladder	18	1 kb DNA Extension Ladder
2	Conventional Control LH244	19	Conventional Control LH244
3	MON 87411	20	MON 87411
4	No template control	21	PV-ZMIR10871
5	Conventional Control LH244	22	No template control
6	MON 87411	23	Conventional Control LH244
7	PV-ZMIR10871 (2 µl)	24	MON 87411
8	No template control	25	PV-ZMIR10871
9	Conventional Control LH244	26	No template control
10	MON 87411	27	Conventional Control LH244
11	PV-ZMIR10871 (2 µl)	28	MON 87411
12	No template control	29	PV-ZMIR10871
13	Conventional Control LH244	30	No template control
14	MON 87411 (10 µl)	31	Conventional Control LH244
15	PV-ZMIR10871	32	MON 87411
16	No template control	33	No template control
17	1 kb DNA Extension Ladder	34	1 kb DNA Extension Ladder

Figure 13 (continued) Analysis of Overlapping PCR Products Across the MON 87411 Insert
Arrows next to the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 kb DNA Extension Ladder (Invitrogen, Grand Island, NY) on the ethidium bromide stained gel.

Sequencing of the MON 87411 Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from the conventional control to examine the insertion site in conventional maize (refer to Figure 7, Step 5). The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5' end of the MON 87411 insert paired with a second primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure 14). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5' and 3' flanking sequences of MON 87411 indicates there was a 118 base pair deletion that occurred during integration of the T-DNA, with the remainder of the flanks in MON 87411 being identical to the conventional control. Such changes are common during plant transformation and these changes presumably resulted from double-stranded break repair mechanisms in the plant during the *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998).

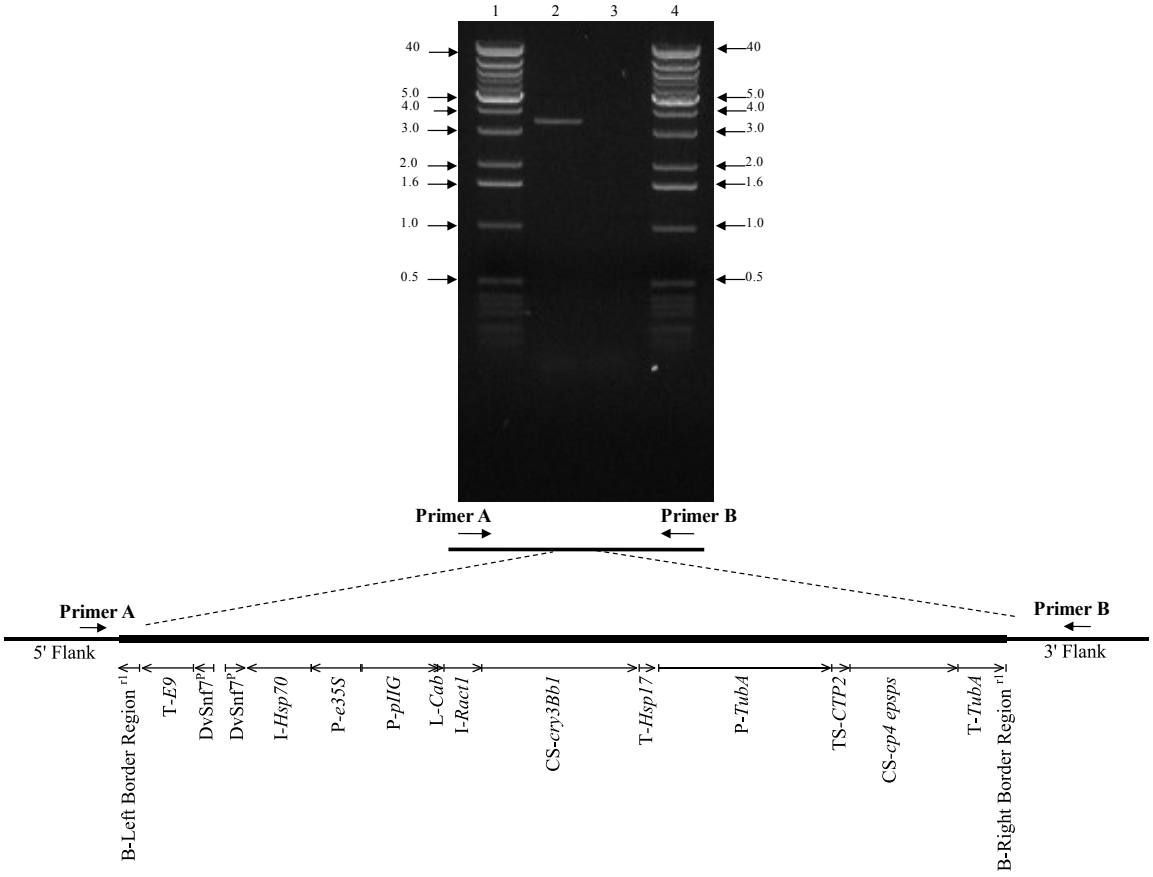


Figure 14 PCR Amplification of the MON 87411 Insertion Site

PCR Analysis was performed to evaluate the MON 87411 insertion site. PCR was performed on conventional control DNA using Primer A, specific to the 5′ flanking sequence, and Primer B, specific to the 3′ flanking sequence of the MON 87411 insert. The DNA generated from the conventional control PCR was used for sequencing analysis. This illustration depicts the MON 87411 insertion site in the conventional control (upper panel) and a schematic of the MON 87411 insert (lower panel). Approximately 5 µl of each of the PCR reactions was loaded on the gel. Lane designations are as follows:

Lane	Sample
1	1 kb DNA Extension Ladder
2	Conventional Control LH244
3	No template DNA control
4	1 kb DNA Extension Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 kb DNA Extension Ladder (Invitrogen, Grand Island, NY) on the ethidium bromide stained gel.

[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.

[P] Superscript in DvSnf7 indicates the partial sequence.

A3(d)(iv) Map of the organisation of the inserted DNA (each site)

PCR and DNA sequence analyses performed on MON 87411 and the conventional control determined the organisation of the genetic elements within the insert as given in Figure 11.

A3(d)(v) Identification and characterisation of unexpected ORFs

The 2009 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2009) includes an assessment element on the identification and evaluation of "open reading frames within the inserted DNA or created by the insertion with contiguous plant genomic DNA." These assessments examine the potential homology of any putative polypeptides or proteins that could be produced from open reading frames (ORFs) in the insert or at the plant-insert junction to known toxins or allergens. These analyses are conducted even if there is no evidence that such alternative reading frames in the insert or such ORFs at the plant-insert junction are capable of being transcribed or translated into a protein. Results from these bioinformatics analyses demonstrate that any putative polypeptides in MON 87411 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

Bioinformatic analyses were performed on the MON 87411 insert and flanking genomic DNA sequences to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by all six reading frames present in the MON 87411 insert DNA as well as ORFs present in the 5' flanking-inserted T-DNA and inserted T-DNA 3' flanking sequence junctions (Figure 15). These various bioinformatic evaluations are depicted in Figure 15. The entire MON 87411 insert DNA sequence was translated in all six reading frames (three forward reading frames and three reading frames in reverse orientation) and the resulting deduced amino acid sequences were then compared to toxin, allergen and all proteins databases using bioinformatic tools. Similarly, ORFs spanning the 5' flanking sequence DNA-inserted DNA junction and inserted DNA-3' flanking sequence DNA junction were translated from stop codon to stop codon in all six reading frames (three forward reading frames and three reading frames in reverse orientation). Putative peptides/polypeptides from each reading frame of eight amino acids or greater were also subjected to bioinformatic analyses. There are no analytical data that indicate any putative polypeptides/proteins subjected to bioinformatic evaluation other than Cry3Bb1 and CP4 EPSPS proteins are produced. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than Cry3Bb1 and CP4 EPSPS was derived from frames 1 to 6 of the insert DNA, or the ORFs spanning the insert junctions; they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications.

Bioinformatics assessment of insert DNA reading frames

Bioinformatic analyses were performed to assess the potential of allergenicity, toxicity, or biological activity of the putative peptides encoded by translation of reading frames 1 through 6 of the inserted T-DNA sequence present in MON 87411 (Figure 15). The FASTA sequence alignment tool was used to assess structural relatedness between the query

sequences and any protein sequences in the AD_2013[3], TOX_2013[4], and PRT_2013[5] databases. Structural similarities shared between the sequence with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity, and the *E*-score. Alignments having *E*-score less than or equal to 1e-5 (1×10^{-5}) are deemed significant because they may reflect shared structure and function among sequences. In addition to structural similarity, the sequence was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope (Silvanovich *et al.*, 2006) and evaluated against the AD_2013 database.

The results of the FASTA and eight amino acid sliding window search comparisons showed that no relevant structural similarity to known allergens were observed for any of the putative polypeptides when compared to proteins in the allergen (AD_2013) database. Using the FASTA algorithm to search the AD_2013 database, no alignments with any of the six query sequences generated an *E*-score of less than or equal to 1e-5. Likewise, no alignment met or exceeded the Codex Alimentarius (2009) FASTA alignment threshold for potential allergenicity of 35% identity over 80 amino acids.

Potential toxicity of sequences translated from the six reading frames was assessed using the FASTA algorithm. Using the FASTA algorithm to search the TOX_2013 database, the translation frame 2 and frame 3 each yielded one alignment below the threshold of 1e-5. The below threshold alignment with the translation of reading frame 2 was with GI-9798640, an 81-kDa leukemia toxin; the alignment displayed 23.596% identity in 445 amino acid overlap. The below threshold alignment with the translation of reading frame 3 was with GI-326429720, a lectoxin-Phi2; the alignment displayed 22.948% identity in 658 amino acid overlap. Inspection of these two alignments revealed that although yielding below threshold *E*-scores, numerous gaps were required to optimize both of the alignments and in addition, the frame 3 translation was punctuated with numerous stop codons. As a result, it is unlikely that either the alignments of the translation of frame 2 or 3 reflect conserved protein structure or therefore toxicity.

When used to query the PRT_2013 database, a comprehensive sequence database containing 27,998,271 sequences, translations of all reading frames yielded alignments with *E*-scores less than or equal to 1e-5. The frame 1 translation positively identified CP4 EPSPS protein

---

[3] The allergen, gliadin, and glutenin sequence database (AD_2013) was obtained from Food Allergy Research and Resource Program Database (FARRP_2013) (http://www.allergenonline.org) and was used for the evaluation of sequence similarities. It is referred to as the AD_2013 database and contains 1,630 sequences.

[4] The toxin database is a subset of sequences derived from the PRT_2013 database that was selected using a keyword search then filtered to remove likely non-toxin proteins. It is referred to as the TOX_2013 database. It contains 8,881 sequences and was used for the evaluation of sequence similarities.

[5] A comprehensive collection of protein sequences that comprises GenBank release 193 was downloaded from NCBI and was used for the evaluation of sequence similarities. It is referred to as the PRT_2013 database and contains 27,998,271 sequences.

(GI-62318479) with a significant *E*-score, 1.4e-160, and is consistent with the known insert structure in MON 87411. The frame 2 translation positively identified Cry3Bb1 protein (GI-77370848) with a significant *E*-score, 0, and is consistent with the known insert structure in MON 87411. The frames 3 and 4 translations yielded numerous alignments with *E*-scores less than 1e-5. Visual inspection of these alignments revealed that the alignments were punctuated with numerous stop codons in the query sequences and required numerous gaps to optimize the alignments. As a result, it is unlikely that the alignments reflect conserved structure. The two significant alignments observed for the frame 5 query sequence were both to "sequence 21505" from two patents. The only annotation associated with the two GenBank entries (GI-228385038 and GI-282789697) described the sequences as being "Nucleic acid and amino acid sequences relating to *Pseudomonas aeruginosa* for diagnostics and therapeutics." Both the alignments display 38% identity over 178 amino acids with an *E*-score of 4.3e-11. Although less than the E-score limit of 1e-5, the alignments with frame 5 displayed less than 40% identity and the annotation associated with the aligned sequences did not provide any indication of toxicity or untoward biological activity. The top alignment yielded using frame 6 translation to search the PRT_2013 database was with unnamed protein product from CaMV (GI-58827) displaying 100% identity in a 82 amino acid overlap with an *E*-score of 4.5e-21. This alignment was not unexpected since it was consistent with the DNA source of the aligned region that was CaMV.

Taken together, these data demonstrate the lack of relevant similarities between known allergens or toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of MON 87411. As a result, in the unlikely event that a translation product other than Cry3Bb1 and CP4 EPSPS was derived from reading frames 1 to 6, these putative polypeptides are not expected to be cross-reactive allergens, toxins, or display adverse biological activity.

Insert junction open reading frame bioinformatics analysis

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the MON 87411 inserted DNA were performed using a bioinformatic comparison strategy (Figure 15). The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) to have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5' flanking sequence DNA, inserted DNA, and the inserted DNA and 3' flanking sequence DNA (Figure 15) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. The resulting putative polypeptides from each reading frame that were eight amino acids or greater in length were compared to AD_2013, TOX_2013, and PRT_2013 databases using FASTA and to the AD_2013 database using an eight amino acid sliding window search. The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and protein sequences in the AD_2013, TOX_2013, and PRT_2013 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and the alignment length (to ascertain if alignments exceeded Codex Alimentarius (2009) threshold of 35% identity in 80

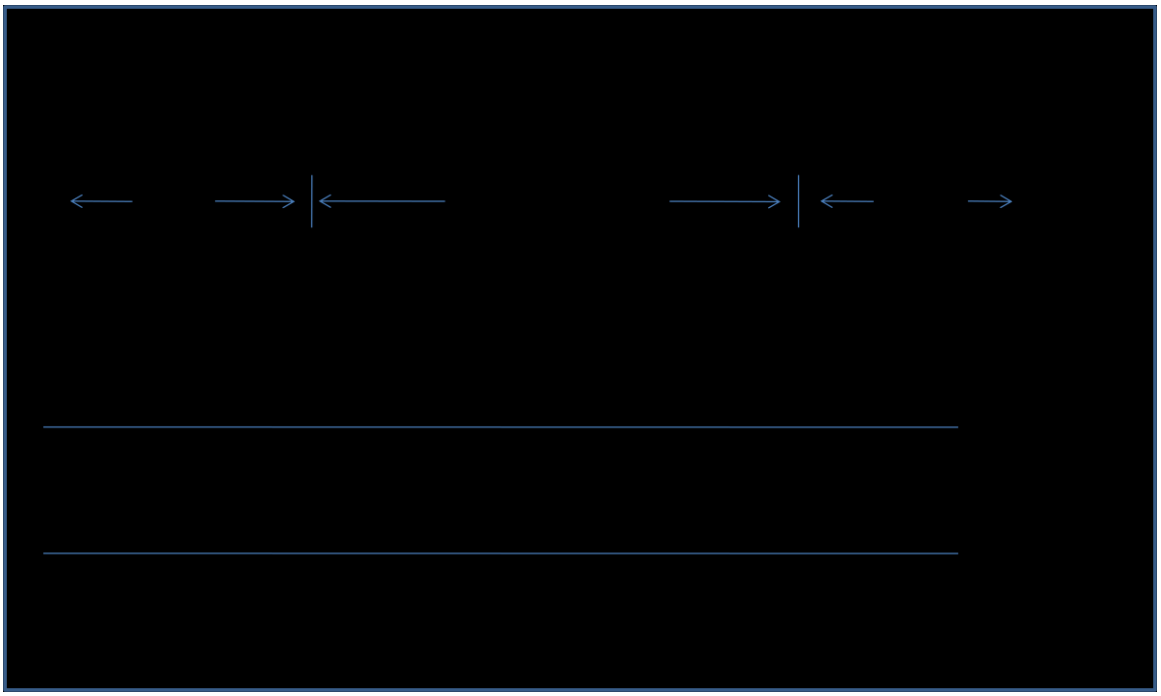
amino acids for FASTA searches of the AD_2013 database), and the *E*-score. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids, as described by Codex (2009), were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope, and evaluated against the AD_2013 database.

The bioinformatic analysis performed using the 8 putative sequences translated from junctions is theoretical as there is no reason to suspect, or evidence to indicate the presence of transcripts spanning the flank junctions. The results of bioinformatic analysis indicate that no structurally relevant sequence similarities were observed between the 8 putative flank junction derived sequences and allergens, toxins or biologically active proteins. As a result, in the unlikely occurrence that any of the 8 putative polypeptides analyzed herein is found *in planta*, none would share significant similarity or identity to known allergens, toxins, or other biologically active proteins that could affect human or animal health.

Bioinformatic assessment of allergenicity, toxicity, and adverse biological activity potential of MON 87411 polypeptides putatively encoded by the insert and flanking sequences summary and conclusions

A conservative bioinformatic assessment of potential allergenicity, toxicity and adverse biological activity for putative polypeptides that were derived from different reading frames of the entire insert or span the 5' and 3' insert junctions was conducted for MON 87411. There are no analytical data that indicate any putative polypeptides/proteins subjected to bioinformatic evaluation are produced other than Cry3Bb1 and CP4 EPSPS. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than Cry3Bb1 and CP4 EPSPS was derived from frames 1 to 6 of the insert DNA, or the ORFs spanning the insert junctions; they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the putative polypeptides for MON 87411 relatedness to known toxins, allergens, or biologically active putative peptides.

Please also refer to Kang and Silvanovich, 2013 (MSL0024715), Kang and Silvanovich, 2013 (MSL0024870), Kang and Silvanovich, 2013 (MSL0024883), Kang and Silvanovich, 2013 (MSL0024900).



AD= AD_2013; TOX= TOX_2013 and PRT= PRT_2013 (GenBank release 193): 8-mer = the eight amino acid sliding window search.

Figure 15 Schematic Summary of MON 87411 Bioinformatic Analyses

A3(e) Family tree or breeding process

The transformation for MON 87411 was conducted with inbred maize line LH244, a patented maize line assigned to Holden's Foundation Seeds LLC in 2001 (U.S. patent #6,252,148). LH244 is a medium season yellow dent maize line with a Stiff Stalk background that is best adapted to the central regions of the U.S. corn belt. LH244 was initiated from a single cross of LH197 × LH199 followed by a backcross to LH197. The F_2 combination ((LH197 × LH197) × LH199) was then selfed and used in the development of LH244.

Following transformation of immature LH244 embryos, a single transformed plant was selected and self-crossed to increase seed supplies. A homozygous inbred line was developed though further self-crossing and selection and was then used to produce other lines which were used for product testing, safety assessment studies, and commercial production. For breeding tree please see Figure 10.

Please also refer to Section A3(f)(i)

A3(f) Evidence of the stability of the genetic changes

A3(f)(i) Pattern of inheritance of insert and number of generations monitored

Determination of insert stability over multiple generations of MON 87411

In order to demonstrate the stability of the T-DNA present in MON 87411 through multiple generations, NGS/JSA analysis was performed using DNA obtained from five breeding generations of MON 87411. The breeding history of MON 87411 is presented in Figure 10, and the specific generations tested are indicated in the figure legend. The MON 87411 (R_4) generation was used for the molecular characterization analyses discussed in Sections A3(d)(i)-A3(d)(iii) and shown in Figure 12. To assess stability, four additional generations were evaluated by NGS/JSA analysis as previously described in Section A3(d), and compared to the fully characterized MON 87411 (R_4) generation. The conventional controls used for the generational stability analysis included LH244, which has a genetic background similar to the MON 87411 (R_4), MON 87411 (R_5) and MON 87411 (R_6) generations and represents the original transformation line. The conventional control hybrid LH244 × HCL645 has a genetic background similar to the MON 87411 R_4F_1 hybrid. In addition, the conventional control hybrid LH244 × LH287, has a genetic background similar to the MON 87411 R_5F_1 hybrid. Genomic DNA isolated from each of the selected generations of MON 87411 and conventional controls were used for NGS/JSA analysis. The results are shown in Figure 16 and Figure 17.

Determination of the insert number

To determine the insert number in the MON 87411 generations, the sequences selected as described in Section A3(d) were analyzed using JSA (Kovalic *et al.*, 2012), where the number of resultant JSCs containing PV-ZMIR10871 DNA sequence determined by this analysis is shown in Table 8 below.

Table 8 Junction Sequence Classes Detected

Sample	JSCs Detected
MON 87411 (R_4)	2
MON 87411 (R_4F_1)	2
MON 87411 (R_5)	2
MON 87411 (R_5F_1)	2
MON 87411 (R_6)	2
LH244	0
LH244 × HCL645	0
LH244 × LH287	0

Figure 16 and Figure 17, below, identify the presence of two, and only two, identical junction sequence classes in each of the five assessed MON 87411 generations (R_4, R_5, R_6, R_4F_1 and R_5F_1) as expected for a stably maintained single insert. This single identical pair of JSCs is observed due to the insertion of PV-ZMIR10871 T-DNA at a single locus in the genome of MON 87411. The consistency of these JSC data across all generations tested demonstrates that this single locus is stably maintained throughout the MON 87411 breeding process.

These results, therefore, demonstrate that the MON 87411 single locus of integration has been maintained through several generations of the breeding of MON 87411; thereby confirming the stability of the insert. Based on this comprehensive sequence data and bioinformatic analysis (NGS/JSA), it is concluded that MON 87411 contains a single and stable T-DNA insertion.

```
R4                -AATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCAT
Directed Seq.     GAATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCAT
R6                GAATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCAT
R4F1              -AATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCAT
R5                GAATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCAT
R5F1              GAATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCAT

R4                TGCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCC
Directed Seq.     TGCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCC
R6                TGCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCC
R4F1              TGCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCC
R5                TGCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCC
R5F1              TGCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCC

R4                TTTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAACC
Directed Seq.     TTTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAACC
R6                TTTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAACC
R4F1              TTTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAAC-
R5                TTTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATG----
R5F1              TTTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGA---
```

Figure 16 Junction Sequences Detected by JSA. Junction Sequence Class A Alignment (All Generations Tested)

Full consensus sequence for JSC-A showing exact alignment to the independently determined *in planta* locus specific sequence (labeled "Directed Seq." in the figure); individual consensus sequences for each of the five generations are labeled according to their generation (R_4, R_5, R_6, R_4F_1 and R_5F_1). Double underlined text indicates plasmid DNA sequence, single underlined text indicates plant genome sequence, and the carat character "^" indicates the junction point between the MON 87411 insert and the flank. Dash characters indicate positions past the end of the consensus sequence for a particular generation.

```
R5F1              AGCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
Directed Seq.     AGCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R5                --CGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R4F1              -GCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R4                ---GGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R6                AGCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA

R5F1              CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
Directed Seq.     CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R5                CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R4F1              CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R4                CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R6                CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT

R5F1              ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTT-----
Directed Seq.     ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R5                ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R4F1              ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R4                ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R6                ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
```

Figure 17 Junction Sequences Detected by JSA. Junction Sequence Class B Alignment (All Generations Tested)

Full consensus sequence for JSC-B showing exact alignment to the independently determined *in planta* locus specific sequence (labeled "Directed Seq." in the figure); individual consensus sequences for each of the five generations are labeled according to their generation (R_4, R_5, R_6, R_4F_1 and R_5F_1). Double underlined text indicates plasmid DNA sequence, single underlined text indicates plant genome sequence, and the carat character "^" indicates the junction point between the MON 87411 insert and the flank. Dash characters indicate positions past the end of the consensus sequence for a particular generation.

Inheritance of the genetic insert in MON 87411

The MON 87411 T-DNA resides at a single locus within the maize genome and therefore should be inherited according to Mendelian principles of inheritance. During development of MON 87411, phenotypic and genotypic segregation data were recorded to assess the inheritance and stability of the MON 87411 T-DNA using Chi-square (χ^2) analysis over several generations. The χ^2 analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 87411 breeding path for generating segregation data is described in Figure 18. The transformed R_0 plant was self-pollinated to generate R_1 seed. An individual homozygous positive plant was identified in the R1 segregating population via a Real-Time TaqMan® PCR assay.

The homozygous positive R_1 plant was self-pollinated to give rise to R_2 seed. The R_2 plants were self-pollinated to produce R_3 seed. The R_3 plants were self-pollinated to produce R_4 seed. Homozygous positive R_4 plants were crossed via traditional breeding techniques to a recurrent parent (HCL645) that does not contain the DvSnf7^p, *cp4 epsps*, or *cry3Bb1* coding sequences to produce hemizygous R_4F_1 seed. The R_4F_1 plants were crossed with the recurrent parent to produce BC_1F_1 seed. The BC_1F_1 generation was tested for the presence of the T-DNA by End-Point TaqMan PCR to select for hemizygous MON 87411 plants. BC_1F_1 plants hemizygous for MON 87411 T-DNA were crossed with the recurrent parent to produce the BC_2F_1 plants. BC_2F_1 plants hemizygous for MON 87411 T-DNA were self-pollinated to produce the BC_2F_2 plants. BC_2F_1 plants hemizygous for MON 87411 T-DNA were crossed with the recurrent parent to produce the BC_3F_1 plants.

The inheritance of the MON 87411 T-DNA was assessed in the BC_2F_1, BC_2F_2, and $BC3F_1$ generations. 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.

A Pearson's Chi-square (χ^2) analysis was used to compare the observed segregation ratios of the MON 87411 T-DNA to the expected ratios. The Chi-square (χ^2) analysis used the statistical program R Version 2.15.2 (2012-10-26).

The Chi-square was calculated as:

$$\chi^2 = \sum [(| o - e |)^2 / e]$$

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where o = observed frequency of the genotype or phenotype and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% (α = 0.05).

The results of the χ^2 analysis of the MON 87411 segregating progeny are presented in Table 9 and Table 10. The χ^2 values in the BC_2F_1 and BC_3F_1 generations indicated no statistically significant difference between the observed and expected 1:1 segregation ratio (hemizygous positive: homozygous negative) of the MON 87411 T-DNA. The χ^2 value for the BC_2F_2 generation indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio (homozygous positive: hemizygous positive: homozygous negative) of MON 87411 T-DNA. These results support the conclusion that the MON 87411 T-DNA resides at a single locus within the maize genome and is inherited according to Mendelian principles. These results are also consistent with the molecular characterization data indicating that MON 87411 contains a single intact copy of the T-DNA inserted at a single locus in the maize genome.

For details please refer to Skottke *et al*., 2013 (MSL0024728).

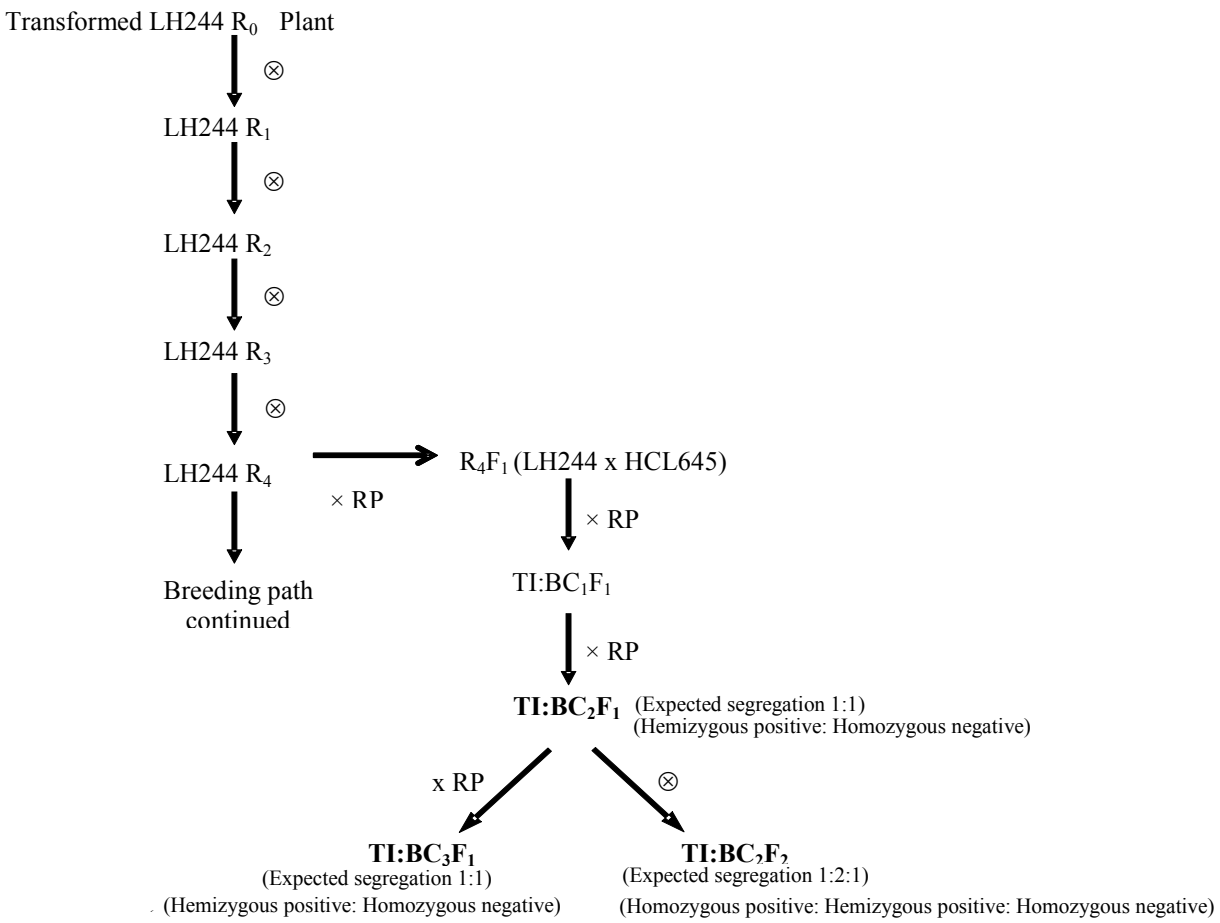

Figure 18 Breeding Path for Generating Segregation Data for MON 87411

*Chi-square analysis was conducted on segregation data from the BC_2F_1, BC_2F_2, and BC_3F_1 generations (bolded text).

TI: Trait Integration: Replacement of genetic background of MON 87411 by recurrent background except inserted gene.

RP: Recurring parent.

⊗=Self-Pollinated

Table 9 Segregation of the T-DNA During the Development of MON 87411 1:1 Segregation

				1:1 Segregation			
Generation	Total Plants	Observed # Plants Positive	Observed # Plants Negative	Expected # Plants Hemizygous (Positive)	Expected # Plants Homozygous Negative	χ^2	Probability[2]
BC_2F_1[1]	351	172	179	175.50	175.50	0.14	0.709
BC_3F_1[1]	223	104	119	111.50	111.50	1.01	0.315

[1] Segregation was evaluated using an End-Point TaqMan analysis for the MON 87411 insert.
[2] Chi-square analysis was performed to analyze the segregation ratios ($p \leq 0.05$).

Table 10 Segregation of the T-DNA During the Development of MON 87411 1:2:1 Segregation

					1:2:1 Segregation				
Generation	Total Plants	Observed # Plants Homozygous Positive	Observed # Plants Hemizygous	Observed # Plants Homozygous Negative	Expected # Plants Homozygous Positive	Expected # Plants Hemizygous	Expected # Plants Homozygous Negative	χ^2	Probability[2]
BC_2F_2[1]	623	152	314	157	155.75	311.50	155.75	0.12	0.942

[1] Segregation was evaluated using Real-Time TaqMan analysis for the MON 87411 insert.
[2] Chi-square analysis was performed to analyze the segregation ratios ($p \leq 0.05$).

Characterization of the genetic modification summary and conclusion

Molecular characterization of MON 87411 by NGS/JSA and directed sequencing demonstrated that a single copy of the intended transfer DNA (T-DNA) containing the DvSnf7 suppression cassette and the *cry3Bb1* and *cp4 epsps* expression cassettes from PV-ZMIR10871 was integrated into the maize genome at a single locus. These analyses also showed no PV-ZMIR10871 backbone DNA had been inserted.

Directed sequence analyses performed on MON 87411 confirmed the organization and intactness of the full T-DNA and all expected elements within the insert, with the exception of incomplete Right and Left Border sequences that do not affect the functionality of the DvSnf7 suppression or *cry3Bb1* and *cp4 epsps* expression cassettes. Analysis of the T-DNA insertion site in maize shows the flanks in MON 87411 are identical to the conventional control, excepting a 118 bp deletion of genomic DNA at the insertion site in MON 87411. This deletion is not expected to affect food or feed safety.

Generational stability analysis by NGS/JSA demonstrated that the T-DNA in MON 87411 was maintained through five breeding generations, thereby confirming the stability of the insert. Results from segregation analyses show heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the presence of the T-DNA in MON 87411 at a single chromosomal locus.

For details please refer to Carleton *et al*., 2013 (MSL0025314).

A3(f)(ii) Pattern of expression of phenotype over several generations

The stability of the T-DNA present in MON 87411 across multiple generations was evaluated by NGS/JSA analyses. Genomic DNA from five generations of MON 87411 (Figure 10) was assayed for all unique junction sequence classes as described above. This information was used to determine the number and identity of insertion sites. For a single insert, two junction sequence classes are expected; each one originates from either end of the insert, both containing portions of DNA insert and flanking sequence. In the case of MON 87411, two identical junction sequence classes were detected in all the generations tested, confirming that the single insert is stably inherited over multiple generations.

Segregation analysis of the T-DNA was conducted to determine the inheritance and stability of the insert in MON 87411. Results from this analysis demonstrate inheritance according to Mendelian principles and the stability of the insert is as expected across multiple generations (Figure 18, Table 7, and Table 8). The segregation analysis corroborates the insert stability demonstrated by NGS/JSA and independently establishes the genetic behavior of the T-DNA as a single chromosomal locus.

For details please refer to Section A3(f)(i).

In order to demonstrate the presence of the Cry3Bb1 and CP4 EPSPS proteins in MON 87411 across multiple breeding generations, western blot analysis of MON 87411 was conducted on

leaf tissue collected from breeding generations R4F1, R4, R5, R5F1, and R6 (Figure 10), and from a conventional control (LH244).

Generational stability of Cry3Bb1 protein expression in MON 87411

The presence of the Cry3Bb1 protein was demonstrated in five breeding generations of MON 87411 using western blot analysis (Figure 19). An *E coli*-produced Cry3Bb1 standard was used as a reference for the identification of the Cry3Bb1 protein. The presence of Cry3Bb1 protein in leaf tissues of MON 87411 was determined by visual comparison of the bands produced in multiple breeding generations (Figure 19, Lanes 6–10) to the Cry3Bb1 reference standard (Figure 19, Lane 4). As expected, the Cry3Bb1 protein was present in all five breeding generations of MON 87411 and migrated with a mobility indistinguishable from that of the *E. coli*-produced protein standard analyzed on the same western blot. Also, as expected, the Cry3Bb1 protein was not detected in the conventional control extract (Figure 19, Lane 5). Additional lower molecular weight protein bands were observed (Figure 19, Lanes 6 - 10). Additional lower molecular weight bands are not unexpected, as Cry3 proteins in plant extracts have previously been shown exist in multiple MW forms (Carroll *et al.*, 1997; Nguyen and Jehle, 2009; Rupar *et al.*, 1991). The specific MW forms present generally dependent on extraction conditions and/or tissue source.

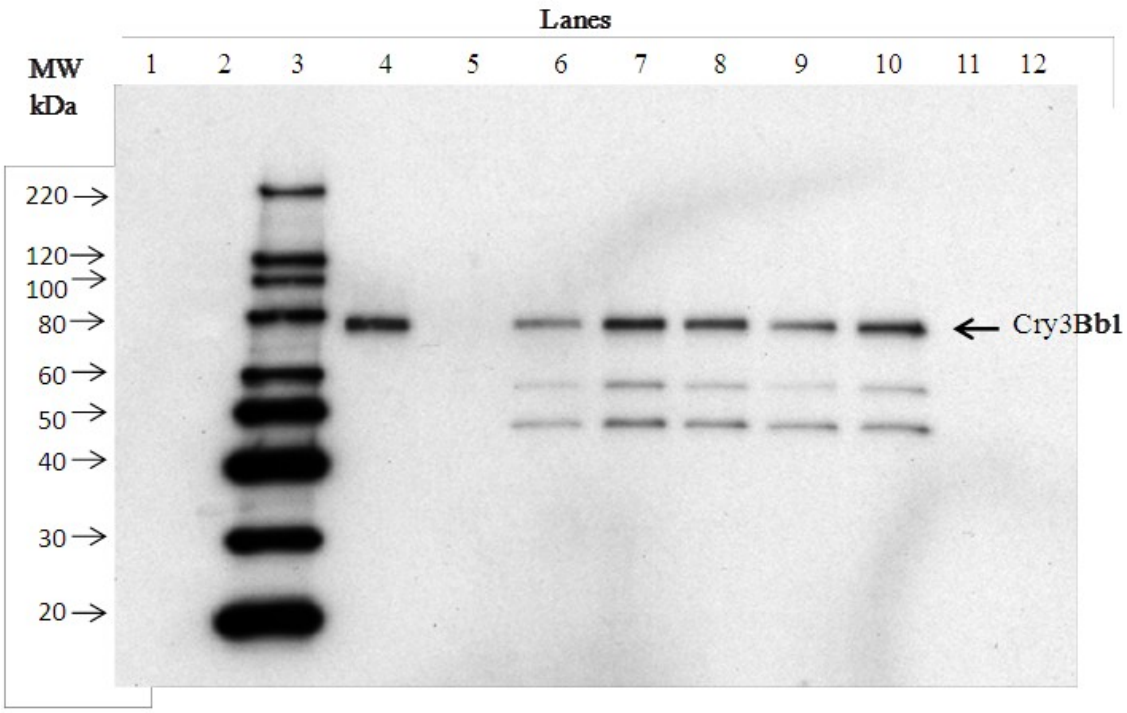

Figure 19 Presence of Cry3Bb1 Protein in Multiple Generations of MON 87411

Extracts from five generations of MON 87411 leaf tissues, conventional control leaf tissue, *E. coli*-produced MON 87411 Cry3Bb1 standard, and molecular weight markers were subjected to SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane. The membrane was incubated with goat anti-Cry3Bb1 antibody and immunoreactive bands visualized through the use of chemiluminescent reagents. The image represents a 5 minutes exposure. Arrows denote the size of the protein, in kiloDaltons (kDa), obtained from Magicmark XP (Invitrogen) transferred to the western membrane. Lane designations are as follows:

Lane	Description	Amount Loaded on Gel
1	Blank	N/A
2	Precision Plus Dual Color Molecular Weight Marker	7 µl
3	MagicMark XP Molecular Weight Marker	10 µl
4	*E. coli*-produced Cry3Bb1 protein (0.4 ng)	20 µl
5	Conventional Substance, Control	20 µl
6	Test Substance, R4F1 generation	20 µl
7	Test Substance, R4 generation	20 µl
8	Test Substance, R5 generation	20 µl
9	Test Substance, R5F1 generation	20 µl
10	Test Substance, R6 generation	20 µl
11	Blank	N/A
12	Blank	N/A

Generational stability of CP4 EPSPS protein expression in MON 87411

The presence of the CP4 EPSPS protein was demonstrated in five breeding generations of MON 87411 using western blot analysis (Figure 20). An *E. coli*-produced CP4 EPSPS standard was used as a reference for the identification of the CP4 EPSPS protein. The presence of CP4 EPSPS protein in leaf tissue of MON 87411 was determined by visual comparison of the bands produced in the multiple breeding generations (Figure 20, Lanes 6–10) to the CP4 EPSPS reference standard (Figure 20, Lane 4). As expected, the CP4 EPSPS protein was present in all five breeding generations of MON 87411 tissue samples and migrated with a mobility indistinguishable from that of the *E. coli*-produced protein standard analyzed on the same western blot. Also, as expected, the CP4 EPSPS protein was not detected in the conventional control extract (Figure 20, Lane 5).

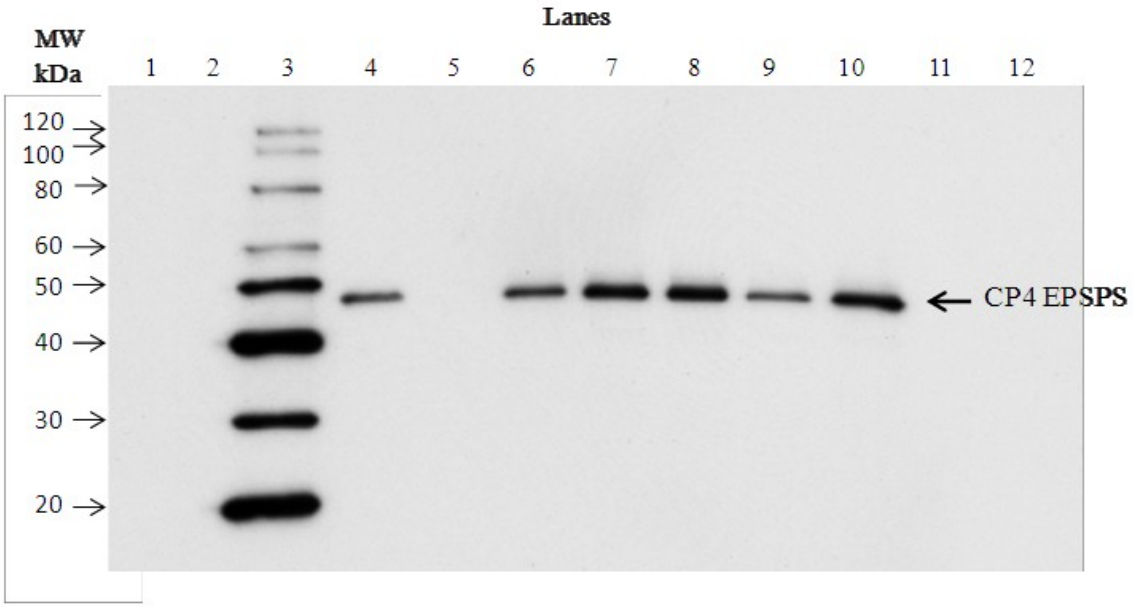

Figure 20 Presence of CP4 EPSPS Protein in Multiple Generations of MON 87411

Aliquots of extracts from five generations of MON 87411 leaf tissues, conventional control leaf tissues, *E. coli*-produced CP4 EPSPS protein standard, and molecular weight markers were subjected to SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane. The membrane was incubated with goat anti-CP4 EPSPS antibody and immunoreactive bands visualized through the use of chemiluminescent reagents. The image represents a 2 minutes exposure. Arrows denote the size of the protein, in kiloDaltons (kDa), obtained from MagicMark XP (Invitrogen) transferred to the western membrane. Lane designations are as follows:

Lane	Description	Amount Loaded on Gel
1	Blank	N/A
2	Precision Plus Dual Color Molecular Weight Marker	7 µl
3	MagicMark XP Molecular Weight Marker	10 µl
4	*E. coli*-produced CP4 EPSPS protein (0.4 ng)	20 µl
5	Conventional Substance, Control	20 µl
6	Test Substance, R4F1 generation	20 µl
7	Test Substance, R4 generation	20 µl
8	Test Substance, R5 generation	20 µl
9	Test Substance, R5F1 generation	20 µl
10	Test Substance, R6 generation	20 µl
11	Blank	N/A
12	Blank	N/A

A4 Analytical Method for Detection

The event-specific DNA-based detection methods such as PCR can be used as the monitoring tool to determine the presence of MON 87411 in a collected sample.

B Information Related to the Safety of the GM Food

B1 Equivalence Studies

B1(a) Cry3Bb1 protein identity and equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of and confirmation of the safety of the introduced protein(s). For the safety data generated using *E. coli*-produced Cry3Bb1 to be applied to Cry3Bb1 protein produced in MON 87411, the equivalence of the plant- and *E. coli*-produced proteins must be assessed. To assess the equivalence between MON 87411-produced Cry3Bb1 and *E. coli*-produced Cry3Bb1 proteins a small quantity of the Cry3Bb1 protein was purified from MON 87411 maize. The Cry3Bb1 protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 87411-produced Cry3Bb1 and the *E. coli*-produced Cry3Bb1 proteins was assessed using a panel of analytical tests, including: 1) N-terminal sequence analysis of the MON 87411-produced Cry3Bb1 protein to establish identity, 2) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of peptides derived from trypsin digested MON 87411-produced Cry3Bb1 protein to establish identity, 3) western blot analysis using anti-Cry3Bb1 polyclonal antibodies to establish identity and immunoreactive equivalence between MON 87411-produced protein and the *E. coli*-produced protein, 4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to establish equivalence of the apparent molecular weight between MON 87411-produced protein and the *E. coli*-produced protein, 5) glycosylation analysis of the MON 87411-produced Cry3Bb1 protein to establish the equivalence between the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins, and 6) Cry3Bb1 activity analysis to demonstrate functional equivalence between MON 87411-produced and the *E. coli*-produced protein. Taken together, these data provide a detailed characterization of the MON 87411-produced Cry3Bb1 protein and establish its equivalence to the *E. coli*-produced Cry3Bb1 protein.

B1(a)(i) Results of the N-terminal sequence analysis of Cry3Bb1

N-terminal sequencing analysis was performed on the MON 87411-produced Cry3Bb1 protein. Two major protein bands were observed in the the western blot analysis (Section B1(a)(iii)) and SDS-PAGE analysis (Section B1(a)(iv)). In addition to analyzing the ~77 kDa band, the expected size of the full length Cry3Bb1 protein, the ~65 kDa band was also analyzed because it is immunoreactive. The reaction did not yield any observable sequence for the ~77.0 kDa band, presumably because the N-terminus was blocked. It is well documented that the N-terminal residue of many proteins is blocked due to post-translational modification *in vivo* (Zybailov *et al.*, 2008). Blocked N-terminal amino acids cannot be directly sequenced by Edman degradation. Although this analysis did not yield N-terminal sequence data, the N-terminus of the ~77.0 KDa band was determined using MALDI-TOF tryptic mass map analysis.

N-terminal sequencing of the first 15 amino acids was performed on the ~65 kDa band of MON 87411-produced Cry3Bb1. The data obtained correspond to the deduced Cry3Bb1 protein sequence beginning at amino acid position 50 (Table 11). Hence, the sequence information confirms the identity of the ~65 kDa Cry3Bb1 band isolated from the grain of MON 87411.

Table 11 N-Terminal Sequence of the MON 87411-produced Cry3Bb1

Amino acid residue # from the N-terminus →	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
Expected Sequence →	T	E	D	S	S	T	E	V	L	D	N	S	T	V	K
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence →	T	E	D	S	S	T	E	V	L	D	N	S	T	V	K

The expected amino acid sequence of the N-terminus of the ~65 kDa Cry3Bb1 protein was deduced from the *cry3Bb1* gene present in MON 87411. The experimental sequences obtained from the MON 87411-produced Cry3Bb1 were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is A, alanine; D, aspatic acid; E, glutamic acid; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; R, arginine; S, serine; T, threonine; V, valine.

B1(a)(ii) Results of MALDI-TOF tryptic mass map analysis of Cry3Bb1

The identity of the MON 87411-produced Cry3Bb1 protein was confirmed by MALDI-TOF MS analysis of peptide fragments produced by a trypsin digestion of the MON 87411-produced Cry3Bb1 protein. In general, protein identification made by proteolytic peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen *et al.*, 1997).

There were 41 unique peptides identified that corresponded to the expected masses of the trypsin-digested sequence of Cry3Bb1 (Table 12). The identified masses were used to assemble a peptide map of the Cry3Bb1 protein (Figure 21). The experimentally determined coverage of the Cry3Bb1 protein was 73% (478 out of 653 amino acids). This analysis further confirms the identity of MON 87411-produced Cry3Bb1 protein. As part of the mass fingerprint analysis, a mass was identified that corresponded to the predicted mass of an acetylated N-terminal tryptic fragment at position 2 of the Cry3Bb1 protein sequence deduced *cry3Bb1* expression cassette. Acetylation of the N-terminus was expected based on the results of the N-terminal sequencing analysis. In addition, removal of the N-terminal methionine is a common modification that occurs prior to acetylation of the nascent protein chain (Giglione and Meinnel, 2001; Van

Damme *et al.*, 2011). Hence, the N-terminus of the ~77.0 kDa MON 87411-produced Cry3Bb1 protein begins with alanine at position two of the expected amino acid sequence and demonstrates that the full length protein is 652 amino acids in length.

Table 12 Summary of the Tryptic Masses Identified for the MON 87411-produced Cry3Bb1 Using MALDI-TOF MS

Observed Mass[1]	Expected Mass	Diff.[2]	Fragment[3]	Sequence[4]
564.33	564.31	0.02	45-48	EFLR
619.35	619.34	0.01	299-303	TELTR
686.45	686.42	0.03	155-160	TPLSLR
727.37	727.35	0.02	2-7	[5]Ac-ANPNNR
794.44	794.40	0.04	394-400	LSFDGQK*
	794.48	0.04	292-298	LYSKGVK*
877.13	876.47	0.66	161-167	SKRSQDR
897.61	897.58	0.03	513-520	ITQLPVVK
925.51	925.47	0.04	573-580	YASTTNLR
937.56	937.53	0.03	348-355	LRPGYFGK
958.58	958.56	0.02	646-653	IEFIPVQL
1026.52	1026.49	0.03	258-266	GSTYDAWVK
1067.58	1067.52	0.06	181-190	NSMP…AVSK
1128.55	1128.56	0.01	377-386	TITS…YGDK
1185.67	1185.70	0.03	556-566	VTLN...LLQR
1350.67	1350.64	0.03	170-180	ELFS...SHFR
1362.72	1362.70	0.02	490-500	GTIP...WTHR
1385.68	1385.66	0.02	501-512	SDVF.…DAEK
1457.84	1457.74	0.10	404-417	TIAN….PNGK
1460.93	1460.86	0.07	554-566	FKVT….LLQR
1496.82	1496.77	0.05	319-331	YGPT….NSIR
1590.97	1590.91	0.06	641-653	IYID…..PVQL
1619.90	1619.83	0.07	168-180	IREL….SHFR
1649.96	1649.84	0.12	626-640	NELI….SNEK
1765.05	1764.98	0.07	304-318	DIFT….TLQK
2001.13	2001.03	0.10	332-347	KPHL….FHTR
2041.22	2041.08	0.14	581-597	LFVQ….YINK
2147.03	2146.93	0.10	472-488	AYSH….MQDR
2298.38	2298.25	0.13	273-291	EMTL….YDIR
2394.23	2394.10	0.13	356-376	DSFN….GSSK
2402.48	2402.35	0.13	191-211	FEVL….LLLK
2483.41	2483.32	0.09	521-545	AYAL…LFLK
2484.26	2484.06	0.20	212-232	DAQV….EFYR
2499.18	2499.08	0.10	425-445	VDFS….YDSK
2552.36	2552.20	0.16	237-257	LTQQ….NGLR
2637.54	2637.29	0.25	131-153	ALAE….NSWK
2655.39	2655.18	0.21	425-446	VDFS…..DSKR
2734.57	2734.28	0.29	447-471	NNGH….PLEK
3444.04	3443.64	0.40	15-44	VTPN…..LNYK*
	3443.71	0.33	404-434	TIAN…...DDQK*
3988.68	3988.93	0.25	15-48	VTPN…..EFLR

[1]The observed mass was collected from at least one of three matrices including α-cyano, DHB and sinapinic acid. The observed mass shown is the mass closest to the expected mass.
[2]The data represent the calculated difference between the expected mass and the observed mass.
[3]Refers to amino acid residue position within the predicted Cry3Bb1 sequence as depicted in Figure 21.
[4]For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are shown separated by dots.
[5]AC is the abbreviation for acetylation. This peptide corresponds to the theoretical N-terminus of the full-length Cry3Bb1 protein minus the first methionine (77 KDa).
*The expected peptide masses are nearly identical (< 1 dalton). Because this analysis could not determine with certainty which expected peptide was actually observed, the peptides with an asterisk (*) were not included in determining sequence coverage (Figure 21).

001 MANPNNRSEH DTIKVTPNSE LQTNHNQYPL ADNPNSTLEE LNYKEFLRMT

051 EDSSTEVLDN STVKDAVGTG ISVVGQILGV VGVPFAGALT SFYQSFLNTI

101 WPSDADPWKA FMAQVEVLID KKIEEYAKSK ALAELQGLQN NFEDYVNALN

151 SWKKTPLSLR SKRSQDRIRE LFSQAESHFR NSMPSFAVSK FEVLFLPTYA

201 QAANTHLLLL KDAQVFGEEW GYSSEDVAEF YRRQLKLTQQ YTDHCVNWYN

251 VGLNGLRGST YDAWVKFNRF RREMTLTVLD LIVLFPFYDI RLYSKGVKTE

301 LTRDIFTDPI FLLTTLQKYG PTFLSIENSI RKPHLFDYLQ GIEFHTRLRP

351 GYFGKDSFNY WSGNYVETRP SIGSSKTITS PFYGDKSTEP VQKLSFDGQK

401 VYRTIANTDV AAWPNGKVYL GVTKVDFSQY DDQKNETSTQ TYDSKRNNGH

451 VSAQDSIDQL PPETTDEPLE KAYSHQLNYA ECFLMQDRRG TIPFFTWTHR

501 SVDFFNTIDA EKITQLPVVK AYALSSGASI IEGPGFTGGN LLFLKESSNS

551 IAKFKVTLNS AALLQRYRVR IRYASTTNLR LFVQNSNNDF LVIYINKTMN

601 KDDDLTYQTF DLATTNSNMG FSGDKNELII GAESFVSNEK IYIDKIEFIP

651 VQL

Figure 21 MALDI-TOF MS Coverage Map of the MON 87411-produced Cry3Bb1

The amino acid sequence of the MON 87411-produced Cry3Bb1 protein was deduced from the *cry3Bb1* gene present in MON 87411. Boxed regions correspond to tryptic peptides that were identified from the MON 87411-produced Cry3Bb1 protein sample using MALDI-TOF MS. In total, 73% (478 out of 652 amino acids) of the expected protein sequence was identified.

B1(a)(iii) Results of western blot analysis of Cry3Bb1 protein

Western blot analysis was conducted using goat anti-Cry3Bb1 polyclonal antibody as additional means to confirm the identity of the MON 87411-produced Cry3Bb1 protein and to assess the equivalence of the immunoreactivity of the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins.

The results showed that an immunoreactive band migrating at the expected apparent MW (~77 kDa) was present in all lanes loaded with the MON 87411-produced (Figure 22, Lanes 10-15) or *E. coli*-produced (Figure 22, Lanes 3-8) Cry3Bb1 proteins. Additional lower molecular weight protein bands, including a predominant ~65 kDa, were observed (Figure 22, Lanes 10-15) in the lanes loaded with MON 87411-produced Cry3Bb1 protein. Additional lower molecular weight bands are not unexpected as Cry3 proteins in plant extracts have previously been shown exist in multiple MW forms (Carroll *et al.*, 1997; Nguyen and Jehle, 2009; Rupar *et al.*, 1991).

To compare the immunoreactivity of the MON 87411-produced and the *E. coli*-produced Cry3Bb1 proteins, densitometric analysis was conducted on bands that migrated to the expected apparent MW for Cry3Bb1 proteins (~77-55 kDa). The signal intensity (reported in OD × mm^2) of the immunoreactive bands identified by anti-Cry3Bb1 antibodies and migrating between the full-length protein at 77.0 kDa and the tryptic core (~55 kDa) were included in the mean signal intensities (Table 13). Because the mean signal intensity of the MON 87411-produced Cry3Bb1 protein band was within 35% of the mean signal intensity of the *E. coli*-produced Cry3Bb1 protein, the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins were determined to have equivalent immunoreactivity.

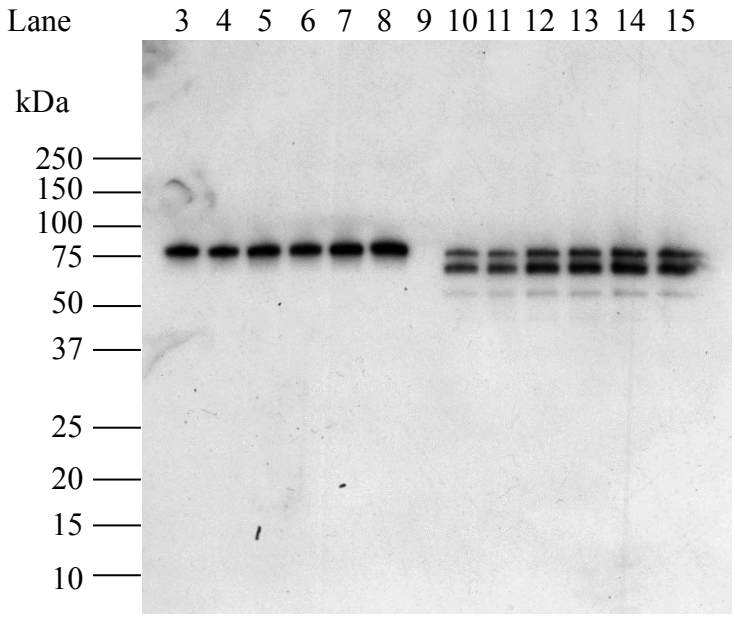

Figure 22 Western Blot Analysis of MON 87411-produced and *E. coli* -produced Cry3Bb1 Proteins

Aliquots of the MON 87411-produced Cry3Bb1 protein and the *E. coli*-produced Cry3Bb1 protein were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. Proteins were detected using anti-Cry3Bb1 antibodies as the primary antibodies. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system. The MWs (kDa) of the standards are shown on the left. Lane 1 and 2 were cropped from the image. The 15 second exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color	
2	Blank	
3	*E. coli*-Produced Cry3Bb1 protein	1
4	*E. coli*-Produced Cry3Bb1 protein	1
5	*E. coli*-Produced Cry3Bb1 protein	2
6	*E. coli*-Produced Cry3Bb1 protein	2
7	*E. coli*-Produced Cry3Bb1 protein	3
8	*E. coli*-Produced Cry3Bb1 protein	3
9	Blank	
10	MON 87411-produced Cry3Bb1 protein	1
11	MON 87411-produced Cry3Bb1 protein	1
12	MON 87411-produced Cry3Bb1 protein	2
13	MON 87411-produced Cry3Bb1 protein	2
14	MON 87411-produced Cry3Bb1 protein	3
15	MON 87411-produced Cry3Bb1 protein	3

Table 13 Comparison of Immunoreactive Signal Between MON 87411-produced and *E. coli*-produced Cry3Bb1Proteins

Mean Signal intensity from MON 87411-produced Cry3Bb1[1] (OD x mm^2)	Mean Signal intensity from *E. coli*-produced Cry3Bb1[1] (OD x mm^2)	Acceptance limits[2] for MON 87411-produced Cry3Bb1(OD x mm^2)
7.35	7.02	4.56-9.48

[1]Each value represents the mean of six values (n=6)

[2] The acceptance limits are for the MON 87411-produced Cry3Bb1 protein and are based on the interval between +35% (7.02 × 1.35) and −35% (7.02 × 0.65) of the mean of the *E. coli*-produced Cry3Bb1 signal intensity across six loads.

B1(a)(iv) Results of the molecular weight analysis of Cry3Bb1 protein

For molecular weight analysis, the MON 87411-produced Cry3Bb1 protein was separated using SDS-PAGE. Following electrophoresis, the gel was stained with Brilliant Blue G-Colloidal stain and analyzed by densitometry (Figure 23). The intact MON 87411-produced Cry3Bb1 protein (Figure 23, Lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced Cry3Bb1 protein (Figure 23, Lane 2) and the apparent MW was calculated to be 77.0 kDa (Table 14). Because the experimentally determined apparent MW of the intact MON 87411-produced Cry3Bb1 protein was within the acceptance limits for equivalence, (Table 14), the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins were determined to have equivalent apparent molecular weights.

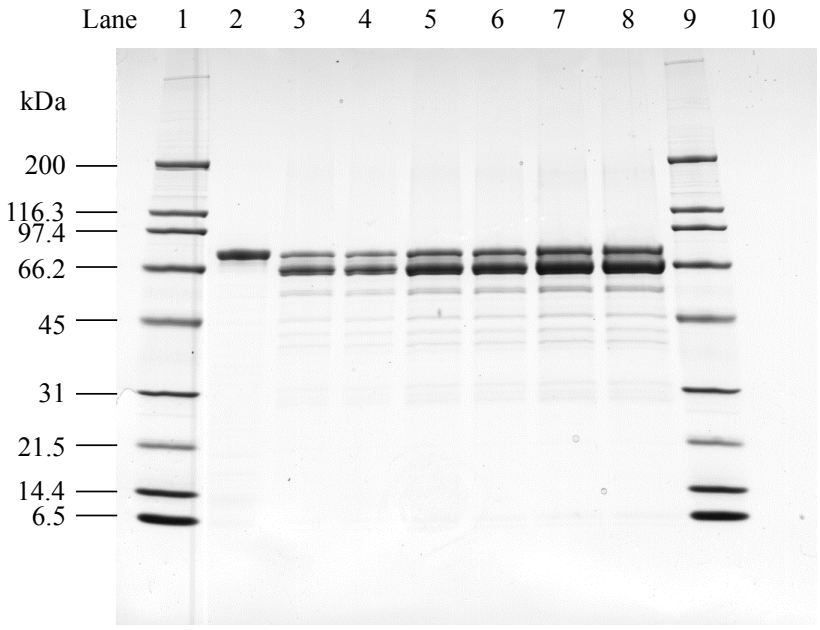

Figure 23 Molecular Weight Analysis of the MON 87411-produced Cry3Bb1 Protein

Aliquots of the MON 87411-produced Cry3Bb1 and the *E. coli*-produced Cry3Bb1 proteins were subjected to SDS-PAGE and gel was stained with Brilliant Blue G-Colloidal stain. The MWs (kDa) are shown on the left and correspond to the standards loaded in Lanes 1 and 9. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standards	4.5
2	*E. coli*-produced Cry3Bb1 protein	1
3	MON 87411-produced Cry3Bb1 protein	1
4	MON 87411-produced Cry3Bb1 protein	1
5	MON 87411-produced Cry3Bb1 protein	2
6	MON 87411-produced Cry3Bb1 protein	2
7	MON 87411-produced Cry3Bb1 protein	3
8	MON 87411-produced Cry3Bb1 protein	3
9	Broad Range MW Standards	4.5
10	Blank	

Table 14 Molecular Weight Comparison Between the MON 87411-produced and *E. coli*-produced Cry3Bb1 Proteins

Apparent MW of MON 87411-produced Cry3Bb1Protein[1] (kDa)	Apparent MW of *E. coli*-produced Cry3Bb1 Protein[2] (kDa)	Preset Acceptance Limits for the MON 87411-produced Cry3Bb1 Protein[3] (kDa)
77.0	74.5	72.9 - 81.7

[1]The apparent MW represents the average of six loadings (n=6) of the MON 87411-produced Cry3Bb1 protein (duplicate loadings of 1, 2 and 3 µgs total protein). The final apparent MW was rounded to one decimal place.
[2]The MW of the *E. coli*-produced Cry3Bb1 protein as reported on its Certificate of Analysis.
[3]Calculated lower and upper bounds for this assay based on two-tailed 95% prediction interval derived from a historical data set of 11 apparent MW determinations for Cry3Bb1.

B1(a)(v) Results of glycosylation analysis for Cry3Bb1 protein

Some eukaryotic proteins can be post-translationally modified with carbohydrate moieties (Rademacher *et al.*, 1988). To test whether Cry3Bb1 protein was glycosylated when expressed in the grain of MON 87411, the MON 87411-produced Cry3Bb1 protein, was subjected to SDS-PAGE, transferred to a membrane and the membrane was analyzed using an ECL™ Glycoprotein Detection Module (GE Healthcare). To assess equivalence of the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins, both the MON 87411- and the *E. coli*-produced Cry3Bb1 protein (previously shown to be free of glycosylation) were analyzed.

A clear glycosylation signal was observed at the expected A clear glycosylation signal was observed at the expected molecular weight (~80kDa) in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure 24, Panel A, Lanes 1-4). In contrast, no glycosylation signals were observed at the expected molecular weight for Cry3Bb1 in the lanes containing the MON 87411-produced Cry3Bb1 protein (Figure 24, Panel A, Lanes 7 and 8) or *E. coli*-produced Cry3Bb1 protein (Figure 24, Panel A, Lanes 9 and 10). The band observed in the *E. coli*-produced Cry3Bb1 sample was at a lower molecular weight than the Cry3Bb1 protein. This band likely represents a naturally biotinylated protein that co-purified with the *E. coli*-produced Cry3Bb1 protein (Choi-Rhee and Cronan, 2003), because it binds to the streptavidin-HRP conjugate and therefore is detectable by the ECL system.

To ensure that the lack of detection of glycosylation of the Cry3Bb1 protein was not due to a lack of protein transferred to the membrane; a second membrane produced in parallel with the membrane analyzed using the ECL™ Glycoprotein Detection Module was stained with Coomassie Blue R250 for protein detection. Both the MON 87411-produced Cry3Bb1 (Figure 24, Panel B, Lanes 7 and 8) and *E. coli*-produced Cry3Bb1 (Figure 24, Panel B, Lanes 9 and 10) proteins were detected; confirming the adequate transfer of the protein. These data indicate that the glycosylation status of MON 87411-produced Cry3Bb1 protein is equivalent to that of the *E. coli*-produced Cry3Bb1 protein and that neither is glycosylated.

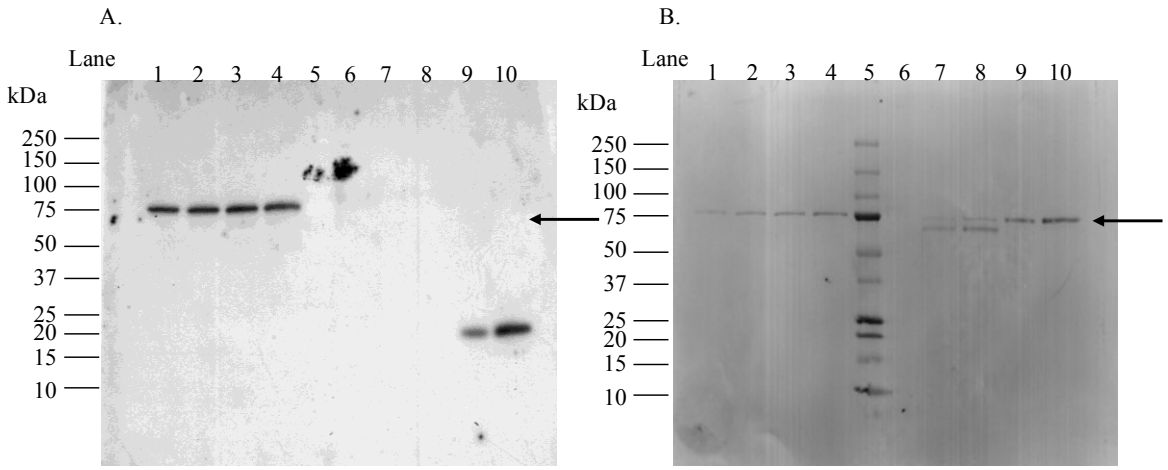

Figure 24 Glycosylation Analysis of the MON 87411-produced Cry3Bb1 Protein

Aliquots of the transferrin (positive control), *E. coli*-produced Cry3Bb1 and MON 87411-produced Cry3Bb1 were subjected to SDS-PAGE and electrotransferred to PVDF membranes. The MWs (kDa) correspond to the Precision Plus Protein™ Dual Color Standards. The arrows show the expected migration of the MON 87411-produced and *E. coli*-produced Cry3Bb1 protein. **Panel A**: Where present, labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based the detection using ECL reagents and exposure to Hyperfilm®. **Panel B**: An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations and the approximate mass loaded are as follows:

Lane	Sample	Amount (ng)
1	Transferrin (positive control)	50
2	Transferrin (positive control)	100
3	Transferrin (positive control)	150
4	Transferrin (positive control)	200
5	Precision Plus Protein™ Standards	
6	Blank	
7	MON 87411-produced Cry3Bb1 protein	100
8	MON 87411-produced Cry3Bb1 protein	200
9	*E. coli*-produced Cry3Bb1 protein	100
10	*E. coli*-produced Cry3Bb1 protein	200

B1(a)(vi) Results of functional activity for Cry3Bb1 protein

The functional activity of the MON 87411-produced and *E. coli*-produced Cry3Bb1 protein was determined by Colorado potato beetle diet-incorporation bioassays. In this assay, activity is expressed as LC_{50}, µg/ml diet. The LC_{50} of the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins were determined to be 0.77 µg/ml diet and 0.67 µg/ml diet; respectively (Table 15). Because the LC_{50} of MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins were both within preset acceptance limits (Table 15), the proteins were determined to have equivalent functional activity.

Table 15 Cry3Bb1 Functional Activity Assay

MON 87411-produced Cry3Bb1 Protein[1] (LC_{50}, µg/ml diet)	E. coli-produced Cry3Bb1 Protein[1] (LC_{50}, µg/ml diet)	Preset Acceptance Limits for MON 87411-produced Cry3Bb1 Protein[2] (LC_{50}, µg/ml diet)
0.77	0.67	0.37 – 0.97

[1]Value refers to mean based on n = 3

[2]Calculated lower and upper bounds for this assay based on two-tailed 95% prediction interval derived from a historical data set of 11 LC_{50} determinations for Cry3Bb1.

B1(a)(vii) Cry3Bb1 protein identity and equivalence conclusion

A panel of analytical techniques was used to characterize the MON 87411-produced Cry3Bb1 protein purified from seed of MON 87411. In addition, the equivalence of the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins was evaluated by comparing their apparent MW, immunoreactivity with anti-Cry3Bb1 antibodies, glycosylation status, and functional activity. The results demonstrate that the MON 87411-produced Cry3Bb1 protein and the *E. coli*-produced Cry3Bb1 protein are equivalent. This demonstration of protein equivalence justifies application of the existing Cry3Bb1 protein safety data set to the Cry3Bb1 protein produced by MON 87411.

For details please refer to Hernan *et al*., 2013 (MSL0024872).

B1(b) CP4 EPSPS protein identity and equivalence

The safety of *E. coli*-produced CP4 EPSPS protein has been assessed previously and the results are summarized by Harrison *et al*. (1996). For the existing CP4 EPSPS safety data set to be applied to CP4 EPSPS protein produced in MON 87411, the equivalence of the plant- and *E. coli*-produced protein must be established. To assess the equivalence between MON 87411-produced and *E. coli*-produced CP4 EPSPS protein, a small quantity of the CP4 EPSPS protein

was purified from MON 87411 maize. The MON 87411-produced CP4 EPSPS protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 87411-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein was assessed using a panel of analytical tests, including: 1) N-terminal sequence analysis of the MON 87411-produced CP4 EPSPS protein to establish identity, 2) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of peptides derived from tryptic digested MON 87411-produced CP4 EPSPS protein to establish identity, 3) western blot analysis using anti-CP4 EPSPS polyclonal antibodies to establish identity and immunoreactive equivalence between MON 87411-produced protein and the *E. coli*-produced protein, 4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to establish equivalence of the apparent molecular weight between MON 87411-produced protein and the *E. coli*-produced protein, 5) glycosylation analysis of the MON 87411-produced CP4 EPSPS protein to establish the equivalence between the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins, and 6) CP4 EPSPS enzymatic activity analysis to demonstrate functional equivalence between MON 87411-produced and the *E. coli*-produced protein. Taken together, these data provide a detailed characterization of the MON 87411-produced CP4 EPSPS protein and establish its equivalence to the *E. coli*-produced CP4 EPSPS protein.

Furthermore, because CP4 EPSPS proteins isolated from other Roundup Ready crops have been demonstrated previously to be equivalent to the *E. coli*-produced CP4 EPSPS protein, by inference, the MON 87411-produced CP4 EPSPS protein is equivalent to the CP4 EPSPS proteins expressed in other Roundup Ready crops, all of which have been reviewed by various regulatory agencies around the world.

B1(b)(i) Results of the N-terminal sequence analysis of CP4 EPSPS protein

N-terminal sequencing was performed on the MON 87411-produced CP4 EPSPS protein. The expected sequence for the CP4 EPSPS protein deduced from the *cp4 epsps* gene present in MON 87411 was observed with the exception of the N-terminal methionine, which was not detected. This result is expected, as removal of the N-terminal methionine, catalyzed by methionine aminopeptidase, is a common modification that occurs co-translationally before completion of the nascent protein chain (Giglione and Meinnel, 2001). The data obtained correspond to the deduced CP4 EPSPS protein beginning at amino acid position 2 (Table 16).

Hence, the sequence information confirms the identity of the CP4 EPSPS protein isolated from the grain of MON 87411.

Table 16 N-Terminal Sequence of the MON 87411-produced CP4 EPSPS Protein

Amino acid residue # from the N-terminus →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Expected Sequence →	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence →	–	L	H	G	A	S	S	R	P	A	T	A	R	K	X	X

The experimental sequences obtained from the MON 87411-produced CP4 EPSPS protein were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is M, methionine; L, leucine; H, histidine; G, glycine; A, alanine, S, serine; R, arginine; P, proline; T, threonine; K, lysine; (X) indicates that the residue was not identifiable.

B1(b)(ii) Results of the MALDI-TOF tryptic mass map analysis of CP4 EPSPS protein

The identity of the MON 87411-produced CP4 EPSPS protein was confirmed by MALDI-TOF MS analysis of peptide fragments produced by the trypsin digestion of the MON 87411-produced CP4 EPSPS protein. In general, protein identification made by proteolytic peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen *et al.*, 1997). There were 23 unique peptides identified that corresponded to the masses expected to be produced by trypsin digestion of the CP4 EPSPS protein (Table 17). The identified masses were used to assemble a coverage map of the entire CP4 EPSPS protein (Figure 25). The experimentally determined mass coverage of the CP4 EPSPS protein was 67.3% (306 out of 455 amino acids).

Table 17 Summary of the Tryptic Masses Identified for the MON 87411-produced CP4 EPSPS Using MALDI-TOF MS

Observed Mass[1]	Expected Mass	Difference[2]	Fragment[3]	Sequence[4]
389.20	389.25	0.05	225-227	TIR
474.23	474.27	0.04	228-231	LEGR
599.33	599.33	0.00	29-33	SISHR
616.33	616.34	0.01	128-132	RPMGR
629.34	629.29	0.05	201-205	DHTEK
711.45	711.45	0.00	133-138	VLNPLR
733.44	733.38	0.06	352-357	VKESDR
835.39	835.39	0.00	62-69	AMQAMGAR
863.47	863.46	0.01	15-23	SSGLSGTVR
872.45	872.45	0.00	313-320	GVTVPEDR*
872.46	872.52	0.06	358-366	LSAVANGLK*
930.49	930.51	0.02	169-177	VPMASAQVK
948.51	948.52	0.01	161-168	TPTPITYR
991.56	991.55	0.01	14-23	KSSG…GTVR
1115.57	1115.57	0.00	295-305	LAGG...ADLR
1357.72	1357.71	0.01	146-157	SEDG...VTLR
1359.67	1359.72	0.05	354-366	ESDR...NGLK*
1359.63	1359.64	0.01	34-46	SFMF...GETR*
1558.82	1558.83	0.01	47-61	ITGL...NTGK
1646.85	1646.84	0.01	389-405	GLGN...LDHR
1763.83	1763.81	0.02	367-382	LNGV...LVVR
1993.97	1993.97	0.00	206-224	MLQG...DGVR
2183.19	2183.17	0.02	275-294	TGLI...INPR
2367.33	2367.33	0.00	178-200	SAVL...IMTR
2450.24	2450.23	0.01	24-46	IPGD...GETR*
	2450.22	0.02	105-127	LTMG...SLTK*
3244.52	3244.52	0.00	73-104	EGDT...TGCR
3249.59	3249.62	0.03	321-351	APSM...EELR
4188.47	4188.26	0.21	234-274	LTGQ...NPTR

[1]The observed mass was collected from at least one of three matrices including α-cyano, DHB and sinapinic acid. The observed mass shown is the mass closest to the expected mass.
[2]The data represent the calculated difference between the expected mass and the observed mass.
[3]Refers to amino acid residue position within the predicted CP4 EPSPS sequence as depicted in Figure 25.
[4]For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are shown separated by dots.
*The expected peptide masses are nearly identical (<1 dalton). Because this analysis did not determine with certainty which expected peptide was actually observed, the peptides with an asterisk (*) were not included in determining sequence coverage (Figure 25).

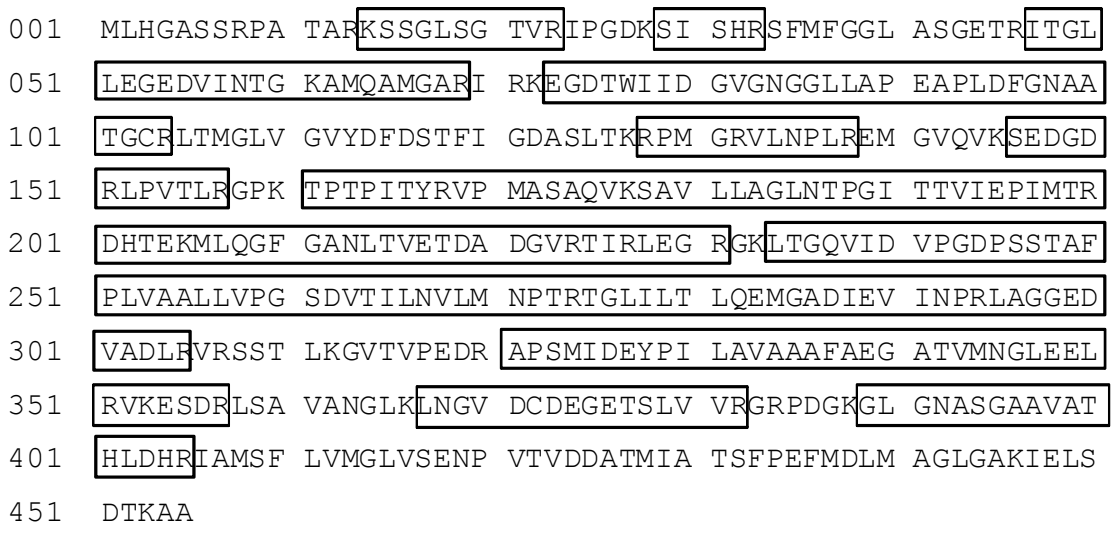

Figure 25 MALDI-TOF MS Coverage Map of the MON 87411-produced CP4 EPSPS Protein

The amino acid sequence of the CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in MON 87411. Boxed regions correspond to regions covered by tryptic peptides that were unambiguously identified from the MON 87411-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 67.3 % (306 of 455 total amino acids) of the expected protein sequence was covered by the identified peptides.

B1(b)(iii) Results of western blot analysis of the CP4 EPSPS protein

Western blot analysis was conducted using goat anti-CP4 EPSPS polyclonal antibody as an additional means to confirm the identity of the CP4 EPSPS protein isolated from the grain of MON 87411 and to assess the equivalence of the immunoreactivity of the MON 87411-produced and the *E. coli*-produced CP4 EPSPS proteins.

The results demonstrated that the anti-CP4 EPSPS antibody recognized the MON 87411-produced CP4 EPSPS protein that migrated to an identical position as the *E. coli*-produced CP4 EPSPS protein (Figure 26). Furthermore, the immunoreactive signal increased with increasing amounts of CP4 EPSPS protein loaded.

To compare the immunoreactivity of the MON 87411-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS proteins, densitometric analysis was conducted on bands that migrated to the expected apparent MW for CP4 EPSPS proteins (~44 kDa). The signal intensity (reported in OD × mm^2) of the band of interest in lanes loaded with the MON 87411-produced and the *E. coli*-produced CP4 EPSPS protein was measured (Table 18). Because signal intensity of the MON 87411-produced CP4 EPSPS protein band was within 35% of the *E. coli*-produced CP4 EPSPS protein, the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were determined to have equivalent immunoreactivity.

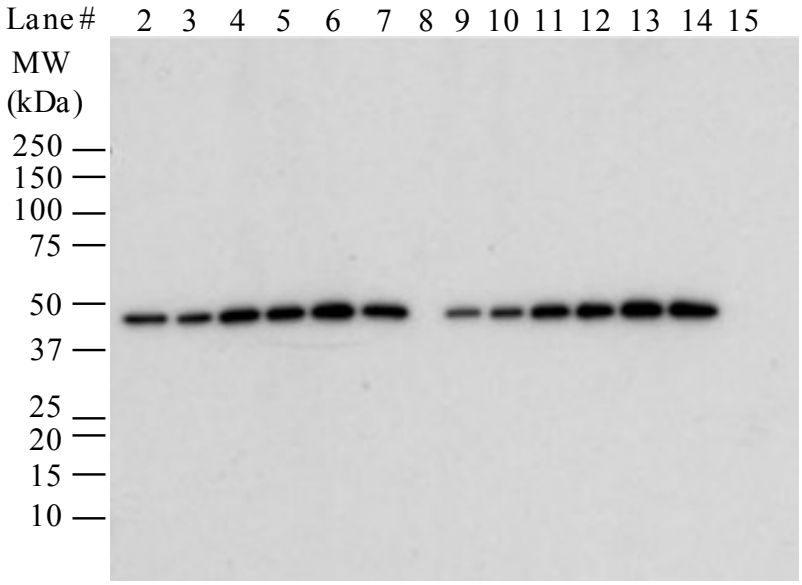

Figure 26 Western Blot Analysis of MON 87411-produced and *E. coli*-produced CP4 EPSPS Proteins

Aliquots of the MON 87411-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-CP4 EPSPS antibodies as the primary antibodies and immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system (GE Healthcare). Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lane 1. Lane 1 was cropped from the image. The 3 min exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color (cropped from the image)	-
2	*E. coli*-produced CP4 EPSPS protein	1
3	*E. coli*-produced CP4 EPSPS protein	1
4	*E. coli*-produced CP4 EPSPS protein	2
5	*E. coli*-produced CP4 EPSPS protein	2
6	*E. coli*-produced CP4 EPSPS protein	3
7	*E. coli*-produced CP4 EPSPS protein	3
8	Blank	-
9	MON 87411-produced CP4 EPSPS protein	1
10	MON 87411-produced CP4 EPSPS protein	1
11	MON 87411-produced CP4 EPSPS protein	2
12	MON 87411-produced CP4 EPSPS protein	2
13	MON 87411-produced CP4 EPSPS protein	3
14	MON 87411-produced CP4 EPSPS protein	3
15	Blank	-

Table 18 Comparison of Immunoreactive Signal Between MON 87411-produced and *E. coli*-produced CP4 EPSPS Protein

Mean Signal Intensity from MON 87411-produced CP4 EPSPS[1] (OD × mm^2)	Mean Signal Intensity from E. coli-produced CP4 EPSPS[1] (OD × mm^2)	Acceptance Limits[2] for MON 87411-produced CP4 EPSPS (OD × mm^2)
6.0	6.0	3.9–8.1

[1]Each value represents the mean of six values (n=6).
[2]The acceptance limits are for the MON 87411-produced CP4 EPSPS protein and are based on the interval between +35% (6.0 × 1.35) and −35% (6.0 × 0.65) of the mean of the *E. coli*-produced CP4 EPSPS signal intensity across six loads.

B1(b)(iv) Results of the molecular weight analysis of the CP4 EPSPS protein

For molecular weight analysis, the MON 87411-produced CP4 EPSPS protein was separated using SDS-PAGE. Following electrophoresis, the gel was stained with Brilliant Blue G-Colloidal stain and analyzed by densitometry (Figure 27). The MON 87411-produced CP4 EPSPS protein (Figure 27, Lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS protein (Figure 27, Lane 2) and the apparent molecular weight was calculated to be 42.9 kDa (Table 19). Because the experimentally determined apparent MW of MON 87411-produced CP4 EPSPS protein was within the preset acceptance limits for equivalence (Table 19; 42.6 – 45.1 kDa), the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were determined to have equivalent apparent molecular weights.

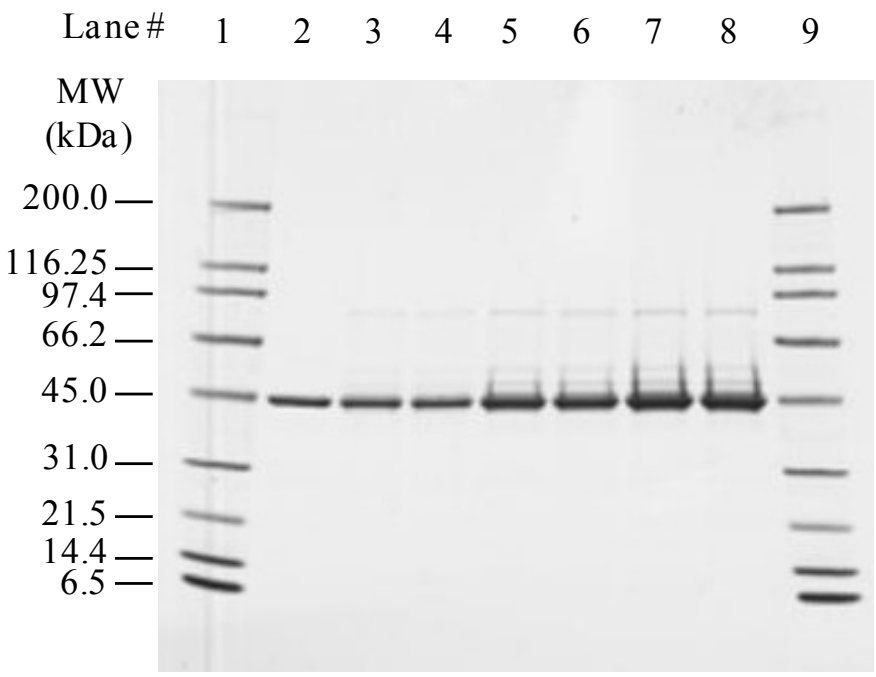

Figure 27 Molecular Weight Analysis of the MON 87411-produced CP4 EPSPS Protein

Aliquots of the MON 87411-produced and the *E. coli*-produced CP4 EPSPS proteins were subjected to SDS-PAGE and the gel was stained with Brilliant Blue G-colloidal stain. The MWs (kDa) are shown on the left and correspond to the standards loaded in Lanes 1 and 9. Lane 10 (blank lane) was cropped from the image. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standards	4.5
2	*E. coli*-produced CP4 EPSPS protein	1
3	MON 87411-produced CP4 EPSPS protein	1
4	MON 87411-produced CP4 EPSPS protein	1
5	MON 87411-produced CP4 EPSPS protein	2
6	MON 87411-produced CP4 EPSPS protein	2
7	MON 87411-produced CP4 EPSPS protein	3
8	MON 87411-produced CP4 EPSPS protein	3
9	Broad Range Molecular Weight Markers	4.5
10	Blank (cropped from the image)	--

Table 19 Molecular Weight Comparison Between the MON 87411-produced and *E. coli*-produced CP4 EPSPS Proteins

Apparent MW of MON 87411-Produced CP4 EPSPS Protein[1] (kDa)	Apparent MW of E. coli-produced CP4 EPSPS Protein[2] (kDa)	Preset Acceptance Limits for MON 87411-produced CP4 EPSPS[3] (kDa)
42.9	43.8	42.6-45.1

[1]The apparent MW represents the average of six loadings (n=6) of the MON 87411-produced CP4 EPSPS protein (duplicate loadings of 1, 2 and 3 µgs total protein). The final apparent MW was rounded to one decimal place.
[2]The MW of the *E. coli*-produced CP4 EPSPS protein as reported on its Certificate of Analysis.
[3]Calculated lower and upper bounds for this assay based on two-tailed 95% prediction interval derived from a historical data set of 16 apparent MW determinations for CP4 EPSPS.

B1(b)(v) CP4 EPSPS glycosylation equivalence

Some eukaryotic proteins can be post-translationally modified with carbohydrate moieties (Rademacher *et al.*, 1988). To test whether the CP4 EPSPS protein was glycosylated when expressed in the grain of MON 87411, the MON 87411-produced CP4 EPSPS protein was subjected to SDS-PAGE, transferred to a membrane and the membrane was analyzed using an ECL™ Glycoprotein Detection Module (GE Healthcare). To assess equivalence of the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins, both the MON 87411- and the *E. coli*-produced CP4 EPSPS proteins (previously been shown to be free of glycosylation) (Harrison *et al.*, 1996) were analyzed.

A clear glycosylation signal was observed at the expected molecular weight in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure 28, Panel A, Lanes 2-5). In contrast, signals were not observed in the lanes containing the MON 87411-produced or *E. coli*-produced protein at the expected molecular weight for the CP4 EPSPS protein (Figure 28, Panel A, Lanes 6-9).

To ensure that the lack of detection of glycosylation of the CP4 EPSPS protein was not due to a lack of protein transferred to the membrane a second control membrane was produced in parallel with the membrane analyzed using the ECL™ Glycoprotein Detection Module and was stained with Coomassie Blue R250 for protein detection, MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were appropriately loaded for glycosylation analysis, (Figure 28, Panel B). Both the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were clearly detected (Figure 28, Panel B, Lanes 6-9). These data indicate that the glycosylation status of MON 87411-produced CP4 EPSPS protein is equivalent to that of the *E. coli*-produced CP4 EPSPS protein and that neither is glycosylated.

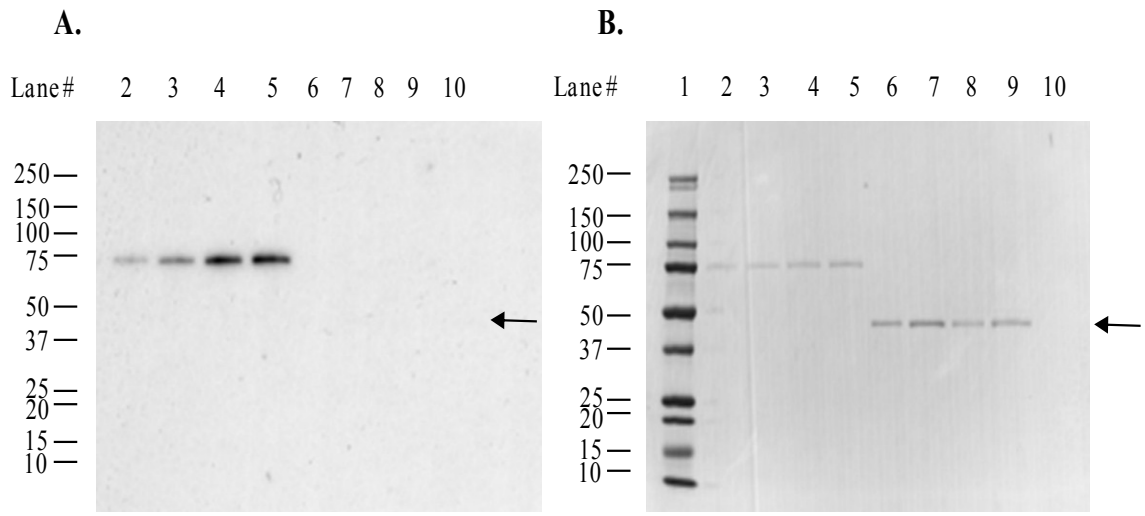

Figure 28 Glycosylation Analysis of the MON 87411-produced CP4 EPSPS Protein

Aliquots of transferrin (positive control), *E. coli*-produced CP4 EPSPS protein and MON 87411-produced CP4 EPSPS protein were subjected to SDS-PAGE and electrotransferred to PVDF membranes. The MWs (kDa) correspond to the Precision Plus Protein™ Standards. The arrows show the expected migration of the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins. **Panel A**: Where present, labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based detection using ECL reagents and exposure to Hyperfilm®. **Panel B**: An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations and the approximate mass loaded are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Transferrin (positive control)	50
3	Transferrin (positive control)	100
4	Transferrin (positive control)	150
5	Transferrin (positive control)	200
6	*E. coli*-produced CP4 EPSPS protein (negative control)	100
7	*E. coli*-produced CP4 EPSPS protein (negative control)	200
8	MON 87411-produced CP4 EPSPS protein	100
9	MON 87411-produced CP4 EPSPS protein	200
10	Blank	-

B1(b)(vi) CP4 EPSPS functional activity

The functional activities of the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were determined using a colorimetric assay that measures formation of inorganic phosphate (Pi) from the EPSPS-catalyzed reaction between shikimate-3-phosphate (S-3-P) and phosphoenolpyruvate (PEP). In this assay, protein-specific activity is expressed as units per milligram of protein (U/mg), where a unit is defined as one µmole of inorganic phosphate released from PEP per minute at 25 °C. The MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were considered to have equivalent functional activity if the specific activities of both were within the acceptance limits of 1.96 to 7.90 U/mg.

The experimentally determined specific activities for the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins are presented in Table 20. The specific activities of MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were 5.78 U/mg and 5.00 U/mg of CP4 EPSPS protein, respectively. Because the specific activity of the MON 87411-produced CP4 EPSPS protein falls within the preset acceptance criterion (Table 20), the MON 87411-produced CP4 EPSPS protein was considered to have equivalent functional activity to that of the *E. coli*-produced CP4 EPSPS protein.

Table 20 CP4 EPSPS Functional Activity Assay

MON 87411-produced CP4 EPSPS Protein[1] (U/mg)	*E. coli*-produced CP4 EPSPS Protein[1] (U/mg)	Previously set Acceptance Limits[2] (U/mg)
5.78 ± 0.19	5.00 ± 0.38	1.96 – 7.90

[1]Value refers to mean and standard deviation calculated based on n=6 which includes three replicate assays spectrophometrically read in duplicate plate wells.
[2]Calculated lower and upper bounds for this assay based on two-tailed 95% prediction interval derived from a historical data set of 22 determinations for CP4 EPSPS.

B1(b)(vii) CP4 EPSPS protein identity and equivalence conclusion

A panel of analytical techniques was used to characterize the MON 87411-produced CP4 EPSPS protein purified from seed of MON 87411. In addition, the equivalence of the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins was evaluated by comparing their apparent MW, immunoreactivity with anti-CP4 EPSPS antibodies, glycosylation status, and functional activity. The results demonstrate that the MON 87411-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein are equivalent. This demonstration of protein equivalence justifies application of the existing CP4 EPSPS protein safety data set to the CP4 EPSPS protein produced by MON 87411.

For details please refer to Lee and Storrs, 2013 (MSL0024834).

B1(c) Characterization and equivalence of DvSnf7 RNA from MON 87411

It is generally known that mRNA which expressed for gene silencing will be broken down in PTGS mechanism (Baulcombe, 2004; Waterhouse and Helliwell, 2003). It is highly unlikely that a dsRNA would produce a protein due to the structural inhibition of scanning ribosome (Kozak, 1989). The DvSnf7 suppression cassette, when transcribed, forms double stranded RNA (dsRNA), which is extremely unlikely to encode for a protein.

B2 Antibiotic Resistance Marker Genes

MON 87411 does not contain genes that encode resistance to antibiotic markers. Molecular characterisation data presented in Section A demonstrate the absence of antibiotic resistance marker gene in MON 87411.

B2(a) Clinical importance of antibiotic that GM is resistant to (if any)

Not applicable.

B2(b) Presence in food of antibiotic resistance protein (if any)

Not applicable.

B2(c) Safety of antibiotic protein

Not applicable.

B2(d) If GM organism is micro-organism, is it viable in final food?

Not applicable.

B3 Characterisation of Novel Proteins or Other Novel Substances

B3(a) Biochemical function and phenotypic effects of novel substances

B3(a)(i) Mode of-action of Cry3Bb1 protein

Cry3Bb1 protein originates from *Bacillus thuringiensis* (Bt), a ubiquitous gram-positive soil bacterium that accumulates crystal proteins during sporulation. Cry3Bb1 protein is a member of the 3D-Cry family of insecticidal proteins (Crickmore, 2012).

The generalized mode-of-action for Cry proteins was described by English and Slatin (1992). It includes ingestion of the crystals by insects and solubilization of the crystals in the insect midgut, followed by activation through proteolytic processing of the soluble Cry protein by digestive enzymes in the midguts. The activated protein then binds to specific receptors on the surface of the midgut epithelium of target insects and inserts into the membrane, leading to pore formation and generalized disruption of the transmembrane gradients and, therefore, cell integrity. While alternate mechanisms have also been proposed, a review of the available data has recently been published and the authors concluded that the original model, pore formation, is the most valid model for Cry protein mode of action (Vachon *et al.*, 2012).

Like other Cry proteins, Cry3Bb1 is synthesized as a prototoxin and is likely cleaved by digestive enzymes in the midgut of target organisms to an approximately 60 activate the protein (Bravo *et al.*, 2007).

B3(a)(ii) Mode-of-action of CP4 EPSPS protein

The enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), catalyzes one of the enzymatic steps of the shikimic acid pathway, and is the target for the broad spectrum herbicide glyphosate (Haslam, 1993; Herrmann and Weaver, 1999; Kishore *et al.*, 1988; Steinrücken and Amrhein, 1980). EPSPS proteins have been isolated from both plant and microbial sources and their properties have been extensively studied (Steinrücken and Amrhein, 1984; Harrison *et al.*, 1996; Haslam, 1993; Schönbrunn *et al.*, 2001). EPSPS enzymes catalyze the transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) (Alibhai and Stallings, 2001).

The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Padgette *et al.*, 1996). In conventional plants, glyphosate blocks the biosynthesis of EPSP (by endogeneous EPSPS), thereby depriving plants of essential amino acids (Haslam, 1993; Steinrücken and Amrhein, 1980). In Roundup Ready plants, which are tolerant to Roundup agricultural herbicides, requirements for aromatic amino acids and other metabolites are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette *et al.*, 1996). The CP4 EPSPS protein expressed in MON 87411 is identical to the CP4 EPSPS protein expressed in Roundup

Ready products across several crops, including soybeans, corn, canola, cotton, sugar beet, and alfalfa.

B3(a)(iii) Mode-of-action of the RNAi component of MON 87411

RNA interference

The RNAi pathway is a natural process in eukaryotic organisms for the regulation of endogenous gene expression (Dykxhoorn *et al.*, 2003; Parrott *et al.*, 2010). The dsRNA molecule that activates the mechanism is first processed by a class of RNase III enzymes called Dicers into small interfering RNAs (siRNAs, ~21-25 nucleotides) (Zamore *et al.*, 2000; Hammond, 2005; Siomi and Siomi, 2009). The resulting siRNA molecules are then incorporated into multiprotein RNA-induced silencing complexes (RISC), which facilitate complementary sequence recognition and mRNA cleavage that leads to specific suppression of the target mRNA (Hammond, 2005; Tomari and Zamore, 2005).

Because of its high specificity and efficacy, the RNAi pathway can be used to control the expression of a gene to attain a specific phenotype or determine a gene's function (Kusaba, 2004). More recently, RNAi via ingestion has been suggested as a potential tool for insect control. Several studies have demonstrated that dsRNAs can be successfully fed to insects either through artificial diet or expressed in transgenic host plants, resulting in mortality of the targeted insects (Baum *et al.*, 2007a; Whyard *et al.*, 2009). The specific suppression of a target mRNA encoding a protein with an essential function within the insect can cause lethality.

Applications of RNAi in plants

Naturally occurring RNA-mediated gene suppression (RNAi) in plants has been previously documented and includes selection for soybean seed coat colour (Tuteja *et al.*, 2004) and maize stalk colour (Della Vedova *et al.*, 2005). In both of these instances, production of chalcone synthase was suppressed leading to significantly decreased pigmentation in soybean seed coats and maize stalks, respectively. In addition, a low glutelin rice variety has been studied and has been determined to result from production of a dsRNA and concomitant suppression of glutelin genes (Kusaba *et al.*, 2003). RNA-mediated gene suppression has also been used in a number of biotechnology-derived food crops that have previously been deregulated by USDA or other regulatory authorities including virus resistant papaya, squash, potato, common bean, and plum as well as a delayed ripening tomato and a soybean with altered oil composition (Parrott *et al.*, 2010). Safety assessments have been conducted (Parrott *et al.*, 2010; Petrick *et al.*, 2013) and global regulatory approvals have been obtained for products employing RNAi gene suppression.

Applications of RNAi in insects

RNAi can also achieve gene silencing in susceptible insects following ingestion of dsRNAs (Baum *et al.*, 2007a; Whyard *et al.*, 2009; Terenius *et al.*, 2011). Insect control products can be developed utilizing RNAi sequence-specific gene silencing to suppress genes critical for insect survival. Because of this sequence-specific gene silencing, these products have the potential to

selectively target a narrow group of closely related pest species and greatly reduce the likelihood of adverse effects on non-target organisms (NTOs), including those beneficial to agriculture. The spectrum of activity for DvSnf7 dsRNA has been shown to be narrow and activity is only evident in a subset of beetles within the Galerucinae subfamily of Chrysomelidae within the Order Coleoptera (Bachman *et al.*, 2013), as described in more detail in Section A1(a).

MON 87411 contains a DvSnf7 suppression cassette that expresses an inverted repeat sequence designed to match the sequence in WCR and thereby utilizes the RNAi pathway to control CRW (*Diabrotica* spp.). The expression of the suppression cassette results in the formation of a dsRNA transcript containing a 240 bp fragment of the WCR *Snf7* gene (DvSnf7). Upon consumption of MON 87411 by WCR, DvSnf7 dsRNA is recognized by the pest's RNAi machinery, resulting in the down-regulation of the targeted DvSnf7 gene leading to WCR mortality (Bolognesi *et al.*, 2012) (Figure 1).

Please see Section A1(a) for further details of Mode of Action of the RNAi Component of MON 87411.

B3(b) Identification of novel substances (e.g. metabolites), levels and site

Cry3Bb1 and CP4 EPSPS protein levels in various tissues of MON 87411 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Tissues of MON 87411 were collected from four replicate plots planted in a randomized complete block field design during the 2011 - 2012 growing season from the following five field sites in Argentina: Pergamino, Buenos Aires (Site Code BAFO); Hunter, Buenos Aires (Site Code BAHT); Pergamino, Buenos Aires (Site Code BAPE); Sarasa, Buenos Aires (Site Code BASS) and Salto, Buenos Aires (Site Code BATC). The field sites were representative of maize-producing regions suitable for commercial maize production. Maize production in the U.S. corn belt and Argentina growing regions occurs at relatively similar latitudes with an approximate 6 month offset (Schnepf *et al.*, 2001). The average growing season temperatures and precipitation are comparable (Schnepf *et al.*, 2001) and, as a result, maize hybrids developed in the U.S. are often used directly by farmers in the southern growing regions of Argentina. As such, protein expression analyses from maize grown at these sites are appropriate for a comparative assessment. Nineteen total tissue samples were collected from each replicated plot at each site, many over several time points/growth stages, throughout the season. Samples included over season leaf (OSL1 through OSL4), over season root (OSR1 through OSR4), over season whole plant (OSWP1 through OSWP4), stover, senescent root, forage root, forage, grain, pollen and silk. MON 87411 plots were treated with glyphosate to generate samples under conditions of the intended use (0.95 kg a.i./ha).

B3(b)(i) Expression levels of Cry3Bb1 protein

Cry3Bb1 protein levels were determined in 19 tissue types. The ELISA results obtained for each sample were averaged across the five sites and are summarized in Table 21. The individual Cry3Bb1 protein levels in MON 87411 across all samples analyzed from all sites

ranged from 3.0 to 460 µg/g dw. The mean Cry3Bb1 protein level among all tissue types was highest in OSWP1 at 340 µg/g dw and lowest in grain at 4.0 µg/g dw.

For details please refer to Beyene, 2013 (MSL0024586).

Table 21 Summary of Cry3Bb1 Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	Cry3Bb1 Mean (SD) Range (µg/g fw)[4]	Cry3Bb1 Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
OSL1	V3-V4	21-22	45 (9.0) 31 – 64	270 (65) 160 - 390	0.035/0.006
OSL2	V6-V8	35-44	40 (7.8) 26 – 56	210 (40) 120 – 270	0.035/0.006
OSL3	V10-V13	50-55	40 (7.9) 21 – 52	170 (35) 92 – 220	0.035/0.006
OSL4	V14-R1	59-78	56 (19) 31 – 89	220 (63) 130 – 340	0.035/0.006
OSR1	V3-V4	21-22	25 (4.6) 16 – 32	180 (43) 130 – 280	0.035/0.028
OSR2	V6-V8	35-44	16 (4.0) 9.4 – 25	120 (24) 67 – 170	0.035/0.028
OSR3	V10-V13	50-55	15 (4.0) 9.6 – 24	84 (21) 54 – 130	0.035/0.028
OSR4	V14-R1	59-78	14 (3.3) 9.0 – 21	75 (19) 43 – 120	0.035/0.028
OSWP1	V3-V4	21-22	44 (4.9) 33 – 53	340 (49) 250 – 460	0.035/0.008
OSWP2	V6-V8	35-44	30 (5.3) 21 – 40	190 (30) 130 – 270	0.035/0.008
OSWP3	V10-V13	50-55	20 (6.8) 9.2 – 33	140 (39) 59 – 210	0.035/0.008
OSWP4	V14-R1	59-78	20 (4.8) 12 – 29	120 (28) 71 – 170	0.035/0.008
Stover	R6	136-155	10 (6.2) 1.9 – 19	21 (13) 4.7 – 44	0.035/0.008
Senescent Root	R6	136-155	4.8 (3.1) 0.76 – 12	19 (13) 3.0 – 50	0.035/0.028

Table 21 (continued) Summary of Cry3Bb1 Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	Cry3Bb1 Mean (SD) Range (µg/g fw)[4]	Cry3Bb1 Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
Forage Root	R5	101-111	7.9 (3.5) 2.6 – 15	36 (16) 13 – 66	0.035/0.028
Forage	R5	101-111	12 (4.9) 5.5 – 23	39 (17) 18 – 75	0.035/0.008
Grain	R6	139-154	3.5 (0.45) 2.7 – 4.4	4.0 (0.56) 3.1 – 5.1	0.035/0.007
Pollen	VT-R1	65-80	29 (3.0) 23 – 34	36 (4.0) 30 – 42	0.035/0.018
Silk	R1	65-81	16 (3.8) 8.5 – 23	160 (37) 89 – 220	0.035/0.010

[1] OSL= over season leaf; OSR= over season root; OSWP= over season whole plant
[2] The crop development stage each tissue was collected.
[3] The number of days after planting that each tissue was collected.
[4] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).
[5] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.
[6] LOQ=limit of quantitation; LOD=limit of detection.

B3(b)(ii) Expression levels of CP4 EPSPS protein

CP4 EPSPS protein levels were determined in all 19 tissue types. The ELISA results obtained for each sample were averaged across the five sites and are summarized in Table 22. The individual CP4 EPSPS protein levels in MON 87411 across all samples analyzed from all sites ranged from less than the limit of quantitation (<LOQ) to 76 µg/g dw. The mean CP4 EPSPS protein level among all tissue types was highest in OSWP1 at 63 µg/g dw and lowest in grain at 1.9 µg/g dw.

For details please refer to Beyene, 2013 (MSL0024586).

Table 22 Summary of CP4 EPSPS Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	CP4 EPSPS Mean (SD) Range (µg/g fw)[4]	CP4 EPSPS Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
OSL1	V3-V4	21-22	7.1 (0.83) 5.8 – 9.0	42 (5.9) 33 – 55	0.137/0.071
OSL2	V6-V8	35-44	7.0 (0.64) 6.0 – 7.9	36 (3.1) 29 – 39	0.137/0.071
OSL3	V10-V13	50-55	7.4 (0.78) 6.4 – 8.9	32 (3.8) 27 – 42	0.137/0.071
OSL4	V14-R1	59-78	7.8 (0.85) 6.6 – 9.5	31 (3.5) 24 – 37	0.137/0.071
OSR1	V3-V4	21-22	6.5 (0.86) 4.4 – 8.0	48 (6.6) 38 – 63	0.068/0.033
OSR2	V6-V8	35-44	5.2 (1.0) 3.8 – 7.1	37 (7.0) 23 – 48	0.068/0.033
OSR3	V10-V13	50-55	5.6 (0.84) 4.0 – 7.1	31 (4.7) 24 – 37	0.068/0.033
OSR4	V14-R1	59-78	5.7 (0.80) 4.2 – 7.1	30 (4.8) 20 – 38	0.068/0.033
OSWP1	V3-V4	21-22	8.1 (0.90) 6.6 – 9.8	63 (6.7) 54 – 76	0.137/0.070
OSWP2	V6-V8	35-44	5.6 (0.94) 3.4 – 7.4	36 (5.8) 21 – 46	0.137/0.070
OSWP3	V10-V13	50-55	4.6 (1.1) 2.3 – 6.6	33 (6.2) 21 – 45	0.137/0.070
OSWP4	V14-R1	59-78	4.3 (0.87) 2.9 – 5.5	25 (5.0) 17 – 32	0.137/0.070
Stover	R6	136-155	1.0 (0.60) 0.30 – 2.1	2.2 (1.2) 0.59 – 4.9	0.137/0.070
Senescent Root	R6	136-155	1.4 (0.69) 0.49 – 2.6	5.4 (2.9) 1.8 – 11	0.068/0.033

Table 22 (continued) Summary of CP4 EPSPS Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	CP4 EPSPS Mean (SD) Range (µg/g fw)[4]	CP4 EPSPS Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
Forage Root	R5	101-111	2.2 (0.81) 1.1 – 4.1	10 (3.7) 5.1 – 19	0.068/0.033
Forage	R5	101-111	2.4 (0.71) 1.5 – 3.8	8.0 (2.3) 5.2 – 13	0.137/0.070
Grain	R6	139-154	1.7 (0.27) 1.4 – 2.7	1.9 (0.31) 1.6 – 3.1	0.228/0.152
Pollen	VT-R1	65-80	15 (1.9) 12 – 19	19 (2.8) 16 – 24	0.137/0.099
Silk	R1	65-81	4.0 (0.69) 3.1 – 5.1	40 (5.0) 32 – 49	0.137/0.121

[1] OSL= over season leaf; OSR= over season root; OSWP= over season whole plant
[2] The crop development stage each tissue was collected.
[3] The number of days after planting that each tissue was collected.
[4] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20, except for stover where n=19 due to one sample expressing <LOQ).
[5] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.
[6] LOQ=limit of quantitation; LOD=limit of detection.

B3(b)(iii) Northern blot analysis of DvSnf7 RNA

The sequence present in the DvSnf7 suppression cassette in MON 87411 was designed to partially match the gene present in western corn rootworm (WCR: *Diabrotica virgifera virgifera*) encoding the SNF7 subunit of the ESCRT-III complex (Babst *et al.*, 2002). Only a portion of the similar sequence in western corn rootworm was utilized in MON 87411 to induce the RNAi mechanism in CRW.

The DvSnf7 suppression cassette contains two 240 bp DvSnf7^{p} sequences in an inverted orientation. There is an intervening sequence of 150 nucleotides between the two DvSnf7^{p} sequences (noted on Table 3 and Table 4). When the suppression cassette is transcribed, the RNA expressed forms a hairpin loop thereby allowing the formation of double stranded DvSnf7 RNA. The DvSnf7^{p} sequences in the suppression cassette produce a 240 bp dsRNA that upon transcription triggers the RNAi mechanism.

Bioinformatic analysis in Section B4 indicates that protein production from DvSnf7 suppression cassette in MON 87411 is highly unlikely.

MON 87411 was analyzed for presence of the full length DvSnf7 transcript as well as generation of the 240 bp dsRNA by RNase I_f digestion from the ≤1.2 kb transcript. Undigested and RNase I_f-digested test and conventional control total RNA samples were hybridized with a probe specific to the *DvSnf7* coding sequence from PV-ZMIR10871. Since the *DvSnf7* coding sequence is present in MON 87411, hybridizing with this probe should result in the detection of hybridization bands on the northern blot. The result of this analysis is shown in Figure 29 .

Conventional control maize total RNA showed no detectable hybridization bands for either undigested (Figure 29, Panel A, Lane 1) or RNase I_f-digested samples (Figure 29, Panel A, lane 4). Undigested total RNA extracted from MON 87411 showed a hybridization band at ~1.2 kb (Figure 29, Panel A, Lane 2). Total RNA extracted from MON 87411 digested with RNase I_f showed a hybridization band at ~240 bases (Figure 29, Panel A, Lane 5). These data indicate that MON 87411 produces the full length ~1.2 kb DvSnf7 transcript and RNase I_f digestion produces the expected 240 base product. Equal loading of the test and control samples was confirmed by ethidium bromide staining of the agarose/formaldehyde gel (Figure 29, Panel B).

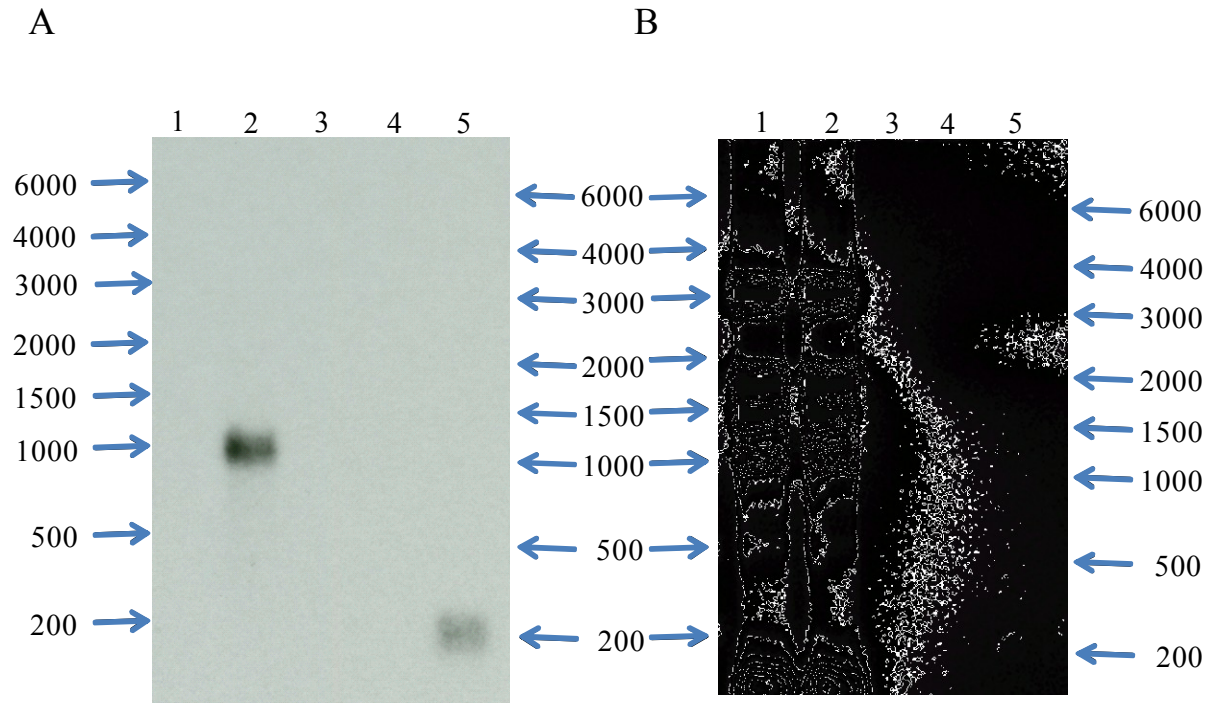

Figure 29 Northern Blot Analysis of MON 87411: DvSnf7 Sequence Probe

A 1.5% agarose/~6.5% formaldehyde gel was loaded with ~10 µg RNA, electrophoresed (B) and then transferred to a nylon membrane for blotting. The blot was hybridized with a ^{32}P-labeled probe specific to the PV-ZMIR10871 *DvSnf7* sequence (A). Lanes 1 and 2 contain undigested total RNA. Lanes 4 and 5 contain RNase I_f-digested total RNA. Lane designations are as follows:

Lane

1 Undigested Conventional Control (LH244)
2 Undigested MON 87411
3 Empty
4 RNase I_f-digest Conventional Control (LH244)
5 RNase I_f-digested MON 87411

The arrows denote the size of the RNA, in bases, obtained from the molecular weight marker on the ethidium bromide stained gel.

B3(c) Site of expression of all novel substances and levels

Please refer to Section B3(b).

B3(d) Post-translational modifications to the novel protein(s)

Not applicable.

B3(e) Evidence of silencing, if silencing is the method of modification

To verify the efficiency of target suppression, DvSnf7 mRNA levels in the insects were measured by real-time RT-PCR. DvSnf7 transcript levels were significantly reduced as early as one day after DvSnf7 dsRNA feeding, and target suppression was even more pronounced in insects feeding on DvSnf7 dsRNA for five days (Figure 30, left). To evaluate if DvSNF7 protein levels also decreased, an anti-SNF7 antibody was generated and used to detect DvSNF7 protein in these larval samples by Western blot (Figure 30, right). This results show that DvSNF7 protein levels were not altered in larvae feeding on DvSnf7 dsRNA for 1 day of exposure. However, DvSNF7 protein levels were significantly reduced in larvae after 5 days of feeding on DvSnf7 dsRNA.

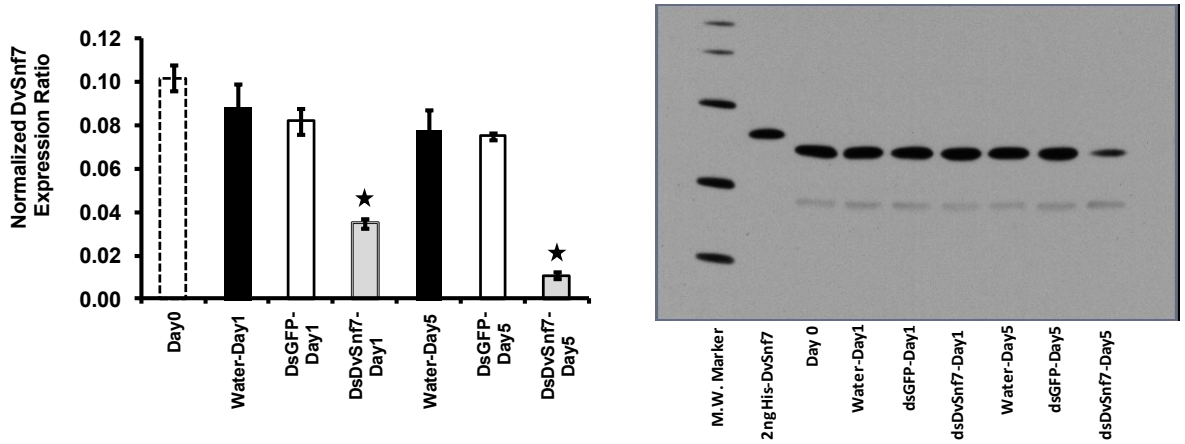

Figure 30 DvSnf7 dsRNA Causes Suppression at mRNA (Left) and Protein Levels (Right) in WCR Larvae (Whole Body) (Bolognesi *et al.*, 2012)

(Left) Real-time RT-PCR results showing significant decrease in DvSnf7 mRNA expression in insects fed with 60 ng/mL of DvSnf7 240 bp dsRNA continuously for one and five days. Insects fed with control diets containing water or GFP (Green Fluorescent Protein) dsRNA do not show mRNA suppression. (Right) Western blot results using anti-SNF7 antibody showed DvSNF7 protein suppression in insects fed with DvSnf7 240 bp dsRNA after 5 days. Stars represent values significantly different from controls ($p = 0.05$; t-test).

B3(f) History of human consumption of novel substances or similarity to substances previously consumed in food

History of safe use of the introduced proteins is a key consideration in the potential for allergenicity and toxicity and for assessment of dietary safety. The history of safe use of Cry3Bb1 and CP4 EPSPS proteins are addressed below.

The assessment of the potential toxicity of introduced proteins is based on comparing the biochemical characteristics of the introduced proteins to characteristics of known toxins. These biochemical characteristics are assessed by determining: 1) if the protein has sequence similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals (Section B4); 2) if the protein is degraded in gastric and intestinal model systems (Section B4); 3) if the protein is stable to heat treatment (Section B4); 4) if the protein exerts any acute toxic effects in mammals (Section B4); and 5) the anticipated exposure levels for humans and animals (Section B5).

B3(f)(i) History of safe use for consumption as food

History of Safe Use of Cry3Bb1 Protein

A history of safe use of Cry3Bb1 protein has been established (Cannon, 1993; WHO, 1999; U.S. EPA, 1988). Microbial pesticides containing *B. thuringiensis* Cry proteins have been used for more than 50 years and subjected to extensive toxicity testing showing no adverse effects to human health (U.S. EPA, 2005; U.S. EPA, 2001a; Baum *et al.*, 1999; Betz *et al.*, 2000; Mendelsohn *et al.*, 2003; McClintock *et al.*, 1995). Cry3Bb1 is one of the active ingredients in the microbial pesticide mixture Raven Oil Flowable Bioinsecticide that was commercialized in the United States in 1995 to control Colorado Potato Beetle in various crops (Baum *et al.*, 1999). Cry3Bb1 is also expressed in commercially available YieldGard VT Rootworm/RR2® corn (MON 88017), YieldGard VT Triple (MON 88017 x MON 810), Genuity® VT Triple PRO (MON 88017 x MON 89034) and Genuity® SmartStax® (MON 89034 × TC1507 × MON 88017 × DAS-59122-7). The amino acid sequence deduced from the Cry3Bb1 expression cassette present in YieldGard VT Rootworm/RR2 (MON 88017) is identical to the amino acid sequence deduced from the Cry3Bb1 expression cassette in MON 87411. A related Cry3Bb1 protein, which has over 99% amino acid identity to the Cry3Bb1 in YieldGard VT Rootworm/RR2 and MON 87411, is expressed in YieldGard Rootworm corn (MON 863). These products expressing the Cry3Bb1 protein have been cultivated in large areas of the U.S. for a number of years. The safe consumption of these crops as food and feed over that time period also demonstrates the history of safe use for Cry proteins as plant-incorporated protectants. Taken together, these data demonstrate that the Cry3Bb1 protein has a history of safe use and does not pose any risk of adverse effects to human and animal health. Additionally, these products have been approved for import to be used as or in food and feed products in many countries around the world including by FSANZ in Australia.

History of Safe Use of CP4 EPSPS Protein

The CP4 EPSPS protein present in MON 87411 is similar to EPSPS proteins consumed in a variety of food and feed sources. CP4 EPSPS protein is homologous to EPSPS proteins naturally present in plants, including food and feed crops (*e.g.,* soybean and maize) and fungal and microbial food sources such as baker's yeast (*Saccharomyces cerevisiae*), all of which have a history of safe consumption (Harrison *et al.*, 1996; Padgette *et al.*, 1996). The ubiquitous presence of homologous EPSPS enzymes in crops and common microorganisms establishes that EPSPS proteins, and their enzymatic activity, pose no hazards for human and animal consumption. In addition, the CP4 EPSPS protein in MON 87411 is identical to the CP4 EPSPS protein in other Roundup Ready crops including Roundup Ready (40-3-2) and Roundup Ready 2 Yield soybeans (MON 89788), Roundup Ready corn 2 (NK603), Roundup Ready canola (RT73), Roundup Ready sugar beet (H7-1), Roundup Ready cotton (MON 1445) and Roundup Ready Flex cotton (MON 88913), Roundup Ready alfalfa (J101 x J163), and YieldGard VT Rootworm/RR2 (MON 88017). Additionally, these products have been approved for import to be used as or in food and feed products in many countries around the world including by FSANZ in Australia.

B3(f)(ii) History of safe use of RNA-mediated gene suppression in plants

RNA-mediated gene suppression (e.g. RNA interference or RNAi) is a naturally occurring, ubiquitous process in eukaryotes, including plants and animals consumed as food and feed. Endogenous RNA-mediated gene suppression is responsible for certain characteristics of conventional crops (Tuteja *et al.*, 2004; Kusaba *et al.*, 2003; Della Vedova *et al.*, 2005) and has also been utilized in some biotechnology-derived crops approved for cultivation and use as food and feed (Ivashuta *et al.*, 2009; Petrick *et al.*, 2013; Parrott *et al.*, 2010). Therefore, there is a history of safe consumption of the RNA molecules mediating gene suppression in plants, including those with homology to genes in humans and other animals (Ivashuta *et al.*, 2009; Jensen *et al.*, 2013). Additionally, there is no evidence to suggest that dietary consumption of nucleic acids is associated with toxicity (Petrick *et al.*, 2013; U.S. FDA, 1992) and U.S. EPA has an established tolerance exemption for nucleic acids that are part of Plant Incorporated Pesticide (PIP) products (U.S. EPA, 2001b). FDA recognizes that all food allergens are proteins (U.S. FDA, 1992; U.S. FDA, 2001) and there is also no evidence of allergenicity of dietary RNA in the peer reviewed scientific literature. The lack of toxicity or allergenicity for ingested RNA also extends to RNA molecules associated with RNA-mediated gene regulation. Therefore an extensive history of safe consumption for dietary RNAs, including dsRNAs, has been established, as reviewed (Petrick *et al.*, 2013). The reason for this history of safe consumption of dietary RNAs is that extensive sequence-independent physiological and biochemical barriers are known to exist in humans and other animals that limit the potential for uptake or activity of ingested nucleic acids (Juliano *et al.*, 2009; Petrick *et al.*, 2013; O'Neill *et al.*, 2011).

Because nucleic acids are a ubiquitous component of the diet for humans and other vertebrates, there are a number of key biological barriers to oral activity of ingested RNA. These barriers

(salivary enzymes, stomach and intestinal acids and enzymes, nucleases in serum, etc.) serve as a general barrier to dietary RNAs entering the systemic circulation, regardless of their nucleic acid sequence (Figure 31).

The first barriers encountered by ingested nucleic acids are salivary RNases (Park *et al.*, 2006) and these nucleic acids subsequently encounter harsh acidic conditions of the stomach that denature and depurinate nucleic acids (Akhtar, 2009; Loretz *et al.*, 2006; O'Neill *et al.*, 2011). Nucleases in the lumen of the gastrointestinal tract/digestive juices and degradative enzymes (and possibly bile salts) from pancreatic secretions into the duodenum also present a significant biological barrier to ingested nucleic acids (Loretz *et al.*, 2006; O'Neill *et al.*, 2011). Any nucleic acids that are absorbed intact must also escape nucleases in the blood (Houck, 1958).

All vertebrate digestive systems display commonalities in regards to structure and function such as enzymes that aid in digestion. As with mammals, the digestive systems of lower vertebrates such as fish, reptiles and birds contain ribonucleases that hydrolyze nucleic acids (Stevens and Hume, 1995). Therefore, the same digestive barriers that prevent oral activity of ingested RNA in mammals are also applicable to lower vertebrates.

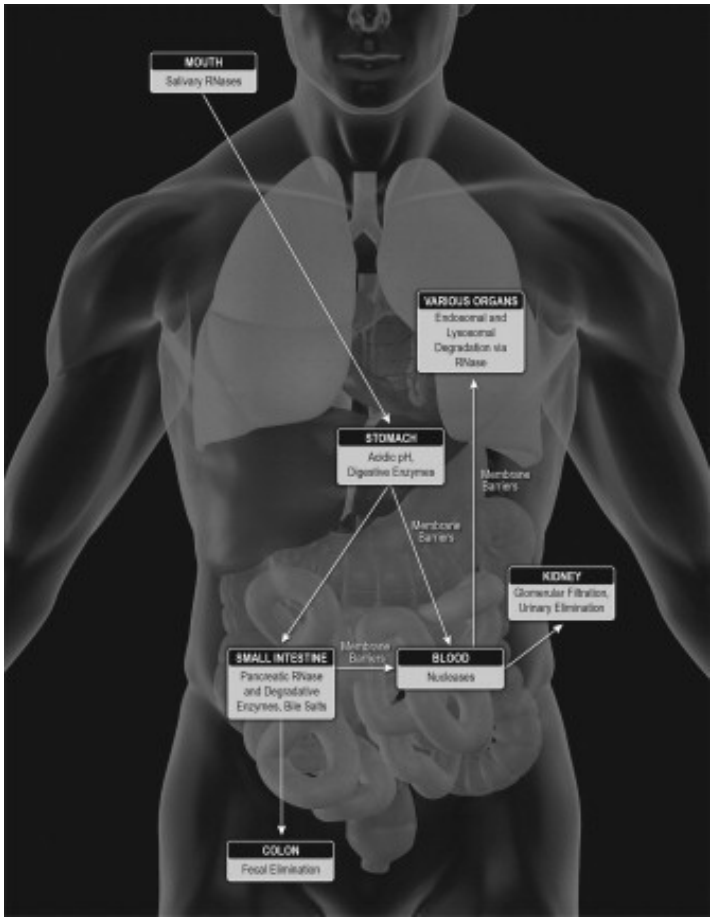

Figure 31 Biological Barriers to Uptake and Activity of Ingested RNA (Petrick et al., 2013)

Cellular membranes of the gut epithelium provide a physical barrier to uptake of high molecular weight, hydrophilic compounds like siRNAs (Akhtar, 2009; Sioud, 2005; O'Neill *et al.*, 2011) and this also applies to longer dsRNAs. Poor cellular uptake of nucleic acids is evidenced by failure of siRNA to suppress gene expression in cultured cells in the absence of transfection reagents at concentrations as high as 250 nM (Lingor *et al.*, 2004). Any small RNAs that pass through cellular membrane barriers must also escape sequestration by endosomes and degradation by lysosomes that limit the activity of exogenous RNA molecules (Sioud, 2005; Gilmore *et al.*, 2004).

Successful RNAi in organisms such as fish, reptiles, and birds has only been achieved *in vitro* and has required the use of transfection agents, direct injection, electroporation, or other invasive techniques (Schyth, 2008; Sifuentes-Romero *et al.*, 2011; Ubuka *et al.*, 2012).

The promise of specificity for oligonucleotide therapeutics created great interest in development of drugs that act through an RNAi mechanism (e.g., RNA oligonucleotide drugs); however, systemic delivery of these therapeutics remains a significant challenge (Behlke, 2006; Nguyen *et al.*, 2008; O'Neill *et al.*, 2011; Vaishnaw *et al.*, 2010). An inability to deliver functional RNA oligonucleotide therapeutics to mediate RNAi is one of the major factors in their limited success and provides empirical evidence that biological barriers limit the activity of exogenous RNA in humans and other mammals. For example, following intravenous injection of unformulated chemically stabilized siRNAs targeting mouse Apolipoprotein B (no delivery agent and not conjugated to a cholesterol tag), efficacy is not observed in the liver of mice following injections as high as 50 mg/kg (Soutschek *et al.*, 2004). This dose level is many orders of magnitude greater than anticipated exposures that would result from consumption of MON 87411. Oligonucleotide therapeutics delivered systemically have succeeded after 1) the introduction of chemical modifications to the oligonucleotides that impart greater stability and 2) formulation in specialized lipophilic delivery vehicles (Behlke, 2006). However, dsRNAs consumed in the diet are not chemically stabilized nor formulated to enable their successful delivery. Consequently, the efficacy and delivery challenges experienced to date with development of oligonucleotide-based human therapeutics provide support for the conclusion that biotechnology-derived crops employing an RNA-mediated gene suppression are safe to consume.

A recent publication reported that ingestion of large doses of a particular small (micro) RNA (miRNA) from rice led to some absorption of the miRNA, detection of the miRNA in serum and liver, and an apparent impact on a target protein and plasma LDL in mice (Zhang *et al.*, 2012a). The authors suggest that a "cross-kingdom" effect – a plant gene product (miR168a) regulating animal gene expression – may be a common phenomenon; and that miRNAs in food may regulate specific genes in animals based upon sequence identity between plant miRNAs and mammalian genes. A second publication (Heinemann *et al.*, 2013), a review article relying almost exclusively on the Zhang (2012a) study, suggests that the current safety/risk assessment approach is not sufficient for RNA-based biotechnology-derived products. As stated in the preceding paragraph, there is no safety concerns related to the consumption of RNA and RNAi in

plants and therefore the current safety assessment approach for dsRNA-containing products, including MON 87411, is appropriate for assessing product safety. The following evidence supports this conclusion:

- Humans regularly consume plants that contain small RNAs. Recent research demonstrates that many existing plant RNAs share sequences with human genes (Ivashuta *et al.*, 2009). This work has also been followed up by studies demonstrating that not only small RNAs but also long dsRNAs in plants share sequence identity to human transcripts (Jensen *et al.*, 2013). Further, humans regularly consume animal-derived foods that are likely to contain more animal miRNAs that have 100% identity to human genes than plant miRNAs. Despite this routine ingestion of plant and animal small RNAs, no impacts on human health have been reported
- A follow up study to Zhang *et al*. (2012a) involving Monsanto scientists (Zhang *et al.*, 2012b) revealed confounding factors which likely explain, in part, the unexpected findings in the 2012 study (Zhang *et al.*, 2012a). In this follow up study, plant miRNAs (including miR168a) were shown to be over-represented relative to their dietary abundance in some public animal small RNA datasets. This indicates that their apparent presence in mouse tissues likely resulted, at least in part, from cross-contamination during the sequencing procedure, thus calling into question the potential for significant uptake of ingested plant miRNAs (Zhang *et al.*, 2012b).
- A recent article exploring the potential role of contamination in the findings of L. Zhang and colleagues provides experimental evidence to support their conclusions that "contamination is, indeed, the underlying cause of these [L. Zhang's] findings" and that their observations "render the conclusions achieved by Zhang and colleagues questionable" (Tosar *et al.*, 2014).
- Differences in diet composition, rather than cross kingdom gene regulation by plant miRNAs, were likely responsible for alterations in plasma LDL cholesterol when Zhang *et al*., (2012a) fed an all-rice diet to mice (Petrick *et al.*, 2013). These results are consistent with the known challenges to oral delivery of nucleic acids (O'Neill *et al.*, 2011).
- In a recent study, Monsanto and miRagen Therapeutics scientists collaborated on a rice feeding study in mice to evaluate claims of dietary miRNA uptake and physiological impact (Dickinson *et al.*, 2013). In this study, miR168a uptake was not reproduced, LDLRAP1 protein levels were unaffected, and LDL was only modulated with a high rice diet and not with a nutritionally balanced rice diet. These results support the conclusion that previously reported observations (Zhang *et al.*, 2012a) were due to variability in gene expression and protein expression data and nutritional differences in animal diets, rather than dietary exposure to miR168a.

- A recent report (Snow *et al.*, 2013) notes the presence of endogenous miRNAs at substantial levels in diets of humans, mice and honey bees. The authors provide empirical data to demonstrate that despite consumption of miRNAs, horizontal delivery via oral ingestion from a typical diet is neither frequent nor prevalent across these consuming organisms. Furthermore, Snow and colleagues demonstrate that dietary small RNA uptake in mammals occurs at levels orders of magnitude lower than that required to initiate RNA-mediated gene suppression
- A recent report (Witwer *et al.*, 2013) conducted a feeding study in nonhuman primates with an miRNA rich food source and based on their results, concluded that, "there is little evidence for presence of these plant miRNA in nonhuman primate blood prior to or following dietary intake of a plant miRNA-rich substance."
- Numerous barriers to the systemic and cellular uptake of exogenous nucleic acids exist (salivary enzymes, stomach and intestinal acids and enzymes, nucleases in serum, etc.) (Petrick *et al.*, 2013; Haupenthal *et al.*, 2006; O'Neill *et al.*, 2011; Akhtar, 2009; Jain, 2008).
- Other authors, organizations, and regulatory bodies have looked specifically at biotechnology-derived RNA-based products and support current risk assessment approaches (ILSI-CERA, 2011; Parrott *et al.*, 2010; FSANZ, 2013).

Finally, these numerous points regarding the ubiquitous nature of RNA in foods, the known barriers to systemic and cellular uptake of exogenous nucleic acids, the known sequence similarity between some plant and human RNAs, and empirical data demonstrating the lack of adverse impacts on non-target organisms lead us to conclude that unique risk issues related to expression of DvSnf7 RNA in MON 87411 do not exist and the current risk/safety assessment approach used by FSANZ is appropriate for assessing this product.

B4 Assessment of Potential Toxicity

The assessment of the potential toxicity of an introduced protein is based on comparing the biochemical characteristics of the introduced protein to characteristics of known toxins. These biochemical characteristics are assessed by determining: 1) if the protein has sequence similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals; 2) if the protein is degraded in gastric and intestinal model systems; 3) if the protein is stable to heat treatment; 4) if the protein exerts any acute toxic effects in mammals; and 5) the anticipated exposure levels for humans and animals. The Cry3Bb1 and CP4 EPSPS proteins in MON 87411 have also been assessed for their potential toxicity as described below.

B4(a) Bioinformatic comparison (aa) of novel protein(s) to toxins

The assessment of the potential for protein toxicity includes bioinformatic analysis of the amino acid sequence of the introduced protein. The goal of the bioinformatic analysis is to ensure that the introduced protein does not share homology to known toxins or anti-nutritional proteins associated with adverse health effects.

Potential structural similarities shared between the CP4 EPSPS or Cry3Bb1 proteins, and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (i.e. primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e. secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. By definition, homologous proteins have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the CP4 EPSPS and Cry3Bb1 amino acid sequences were performed with the toxin database to identify possible homology with proteins that may be harmful to human and animal health. The toxin database, TOX_2013, is a subset of sequences derived from the PRT_2013 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. The TOX_2013 database contains 8,881 sequences.

An *E*-score acceptance criteria of $1x10^{-5}$ or less for any alignment was used to identify proteins from the TOX_2013 database with potential for significant shared structural similarity and function with CP4 EPSPS or Cry3Bb1 proteins. As described above, the *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of 1×10^{-5} or less to be considered to have sufficient sequence similarity to infer homology. The results of the search comparisons showed that no relevant alignments were observed between CP4 EPSPS and protein sequences in the TOX_2013 database. For Cry3bb1 a single alignment with the TOX_2013 database displaying an *E*-score of 3.1×10^{-7}, and 24% identity in

a window of 342 amino acids was obtained with GI-9798640 an 81-kDa parasporin protein from *Bacillus thuringiensis* that binds to leukemia cells. Inspection of the alignment revealed that 14 gapped regions spanning a total of 44 amino acids were required to optimize the alignment. As a result, it is unlikely this alignment reflects conserved structure and the potential of mammalian toxicity

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the CP4 EPSPS or Cry3Bb1 proteins and any known toxic or other biologically active proteins that would be harmful to human or animal health.

For details please refer to Kang and Silvanovich, 2013 (MSL0024870) and Kang Silvanovich, 2013 (MSL0024715)

B4(b) Stability to heat or processing and/or degradation in gastric model

B4(b)(i) Digestibility of the Cry3Bb1 and CP4 EPSPS proteins

The stability of a protein to degradation in simulated mammalian gastrointestinal fluids is a key consideration in the assessment of its potential toxicity. The digestibility of the Cry3Bb1 and CP4 EPSPS proteins was evaluated by incubation with simulated gastric fluids, and the results show that both the Cry3Bb1 and CP4 EPSPS proteins were readily digested (Section B5(c)(i)). Therefore, it is anticipated that exposure to functionally active Cry3Bb1 and CP4 EPSPS protein from the consumption of MON 87411 or foods derived from MON 87411 will be negligible.

B4(b)(ii) Heat stability of Cry3Bb1 and CP4 EPSPS proteins

B4(b)(ii)1 Heat stability of the purified Cry3Bb1 protein

Heat treatment is widely used in maize grain processing and in the preparation of foods containing components derived from maize grain. The effect of heat treatment on Cry3Bb1 and CP4 EPSPS proteins was evaluated using a functional assay to assess the impact of temperature on activity and using SDS-PAGE to assess the impact of temperature on protein integrity. The effect of heating on the functional activity of Cry3Bb1 protein for 15 min and 30 min is presented in Table 23 and Table 24, respectively. After treatment at temperatures of 55 ºC or above and at both time points an 87% or greater loss of detectable functional activity was observed. The effect of heating on band intensity, as measured by SDS-PAGE, of the Cry3Bb1 protein for 15 min and 30 min is presented in Figure 32 and Figure 33, respectively. Heat treatment of the Cry3Bb1 protein incubated at 25, 37 and 55 ºC at both 15 and 30 minutes, had no effect on the band intensity of Cry3Bb1protein. Heating at 75 ºC for 15 minutes had no effect on band intensity; however, incubation for 30 minutes resulted in a small change in Cry3Bb1 protein band intensity. Heating of the Cry3Bb1protein at 95 ºC for either 15 or 30 minutes resulted in significantly reduced Cry3Bb1 protein band intensity, to less than or equal to that of the 10 % Cry3Bb1 protein reference equivalent. These data demonstrate that Cry3Bb1 protein behaves with a predictable tendency towards loss of functional activity at elevated temperatures.

For details please refer to Hernan *et al*., 2011 (MSL0023328) and Hernan *et al*., 2011 (MSL0023307).

Table 23 LC_{50} Values of Cry3Bb1 Activity Following Heat Treatment for 15 Minutes

Temperature, ºC	LC_{50}[1] (µg Cry3Bb1/ml diet)	% Relative Activity[3,4]
0 ºC (control substance)	0.83	100%
25 ºC	0.99	84%
37 ºC	0.86	97%
55 ºC	6.7	12%
75 ºC	> 7.0[2]	< 12%
95 ºC	> 7.0[2]	< 12%

[1]Determined from concentration response curves that included seven Cry3Bb1 concentrations ranging from 0.11 – 7.0 µg Cry3Bb1 protein/ml diet. Each Cry3Bb1 concentration contained one replicate with a target number of twenty-four larvae.
[2] 7.0 µg Cry3Bb1/ml diet represents the highest concentration tested.
[3] No Heat Control was assigned as 100 % activity.
[4] Relative Activity = (0.83 µg Cry3Bb1/ml diet ÷ Heat treatment LC_{50} µg Cry3Bb1/ml diet) * 100

Table 24 LC_{50} Values of Cry3Bb1 Activity Following Heat Treatment for 30 Minutes

Temperature, ºC	LC_{50}[1] (µg Cry3Bb1/ml diet)	% Relative Activity[3,4]
0 ºC (control substance)	0.89	100%
25 ºC	0.74	120%
37 ºC	0.95	94%
55 ºC	> 7.0[2]	< 13%
75 ºC	> 7.0[2]	< 13%
95 ºC	> 7.0[2]	< 13%

[1]Determined from concentration response curves that included seven Cry3Bb1 concentrations ranging from 0.11 – 7.0 µg Cry3Bb1 protein/ml diet. Each Cry3Bb1 concentration contained one replicate with a target number of twenty-four larvae.
[2] 7.0 µg Cry3Bb1/ml diet represents the highest concentration tested.
[3] No Heat Control was assigned as 100 % activity.
[4] Relative Activity = (0.89 µg Cry3Bb1/ml diet ÷ Heat treatment LC_{50} µg Cry3Bb1/ml diet) * 100

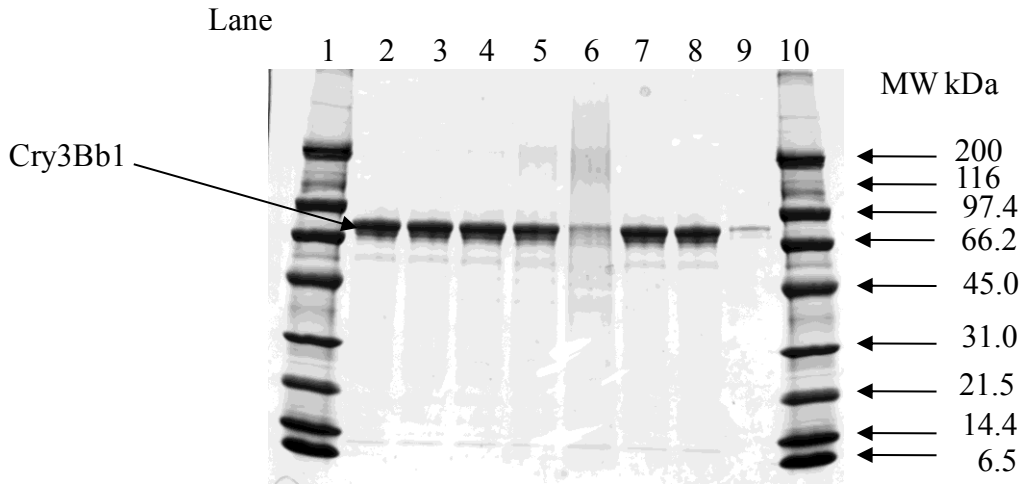

Figure 32 SDS-PAGE of Cry3Bb1 Protein Following Heat Treatment for 15 Minutes

Heat-treated samples of Cry3Bb1 (3.2 µg total protein) subjected to SDS-PAGE and stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Lane designations are as follows:

Lane	Description	Amount (µg)
1	Broad Range MWM	40.5
2	Cry3Bb1 25 ºC	3.2
3	Cry3Bb1 37 ºC	3.2
4	Cry3Bb1 55 ºC	3.2
5	Cry3Bb1 75 ºC	3.2
6	Cry3Bb1 95ºC	3.2
7	Cry3Bb1 Unheated Control (0 ºC)	3.2
8	Cry3Bb1Reference	3.2
9	Cry3Bb1Reference	0.32
10	Broad Range MWM	40.5

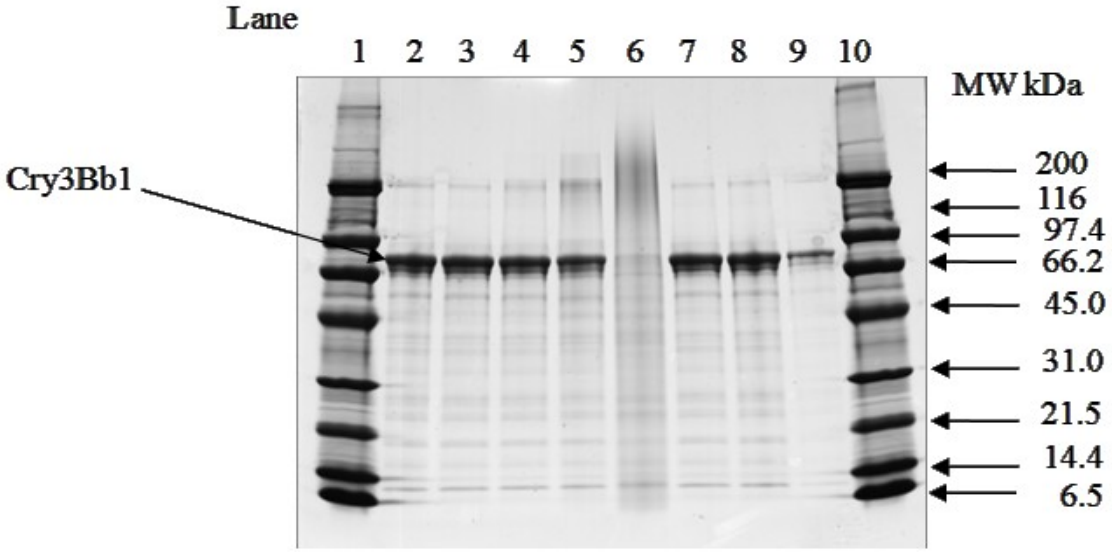

Figure 33 SDS-PAGE of Cry3Bb1 Protein Following Heat Treatment for 30 Minutes

Heat-treated samples of Cry3Bb1 (3.2 µg total protein) subjected to SDS-PAGE and stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Lane designations are as follows:

Lane	Description	Amount (µg)
1	Broad Range MWM	40.5
2	Cry3Bb1 25 ºC	3.2
3	Cry3Bb1 37 ºC	3.2
4	Cry3Bb1 55 ºC	3.2
5	Cry3Bb1 75 ºC	3.2
6	Cry3Bb1 95ºC	3.2
7	Cry3Bb1 Unheated Control (0 ºC)	3.2
8	Cry3Bb1Reference	3.2
9	Cry3Bb1Reference	0.32
10	Broad Range MWM	40.5
1	Broad Range MWM	40.5

B4(b)(ii)2 Heat stability of the purified CP4 EPSPS protein

The effect of heating on the functional activity of the CP4 EPSPS protein for 15 min and 30 min is presented in Table 25 and Table 26, respectively. After treatment at temperatures of 75 ºC and higher, CP4 EPSPS functional activity was below the limit of detection. There was no effect on band intensity, as measured by SDS-PAGE, of heat-treated samples after incubation for 15 or 30 minutes at all temperatures tested (Figure 34 and Figure 35, respectively). These data demonstrate that CP4 EPSPS behaves with a predictable tendency toward enzyme denaturation at elevated temperatures.

Table 25 Activity of CP4 EPSPS after 15 Minutes at Elevated Temperatures

Temperature	Functional Activity CP4 EPSPS (U/mg) (Mean[1])	Relative activity[2]
Unheated Control (0 ºC)	6.03	100%
25 ºC	4.88	81%
37 ºC	5.08	84%
55 ºC	4.22	70%
75 ºC	< LOD[3]	< 3%[4]
95 ºC	< LOD[3]	< 3%[4]

[1] Mean specific activity determined from n = 2 for each treated sample (one at each temperature and time point),.
[32]CP4 EPSPS activity of unheated control was assigned 100 %.
[3] LOD is defined as the value that is three standard deviations above the mean of the assay blank.
[4] Calculated from the LOD of the CP4 EPSPS activity assay.

Table 26 Activity of CP4 EPSPS after 30 Minutes at Elevated Temperatures

Temperature	Functional Activity CP4 EPSPS (U/mg) (Mean[1])	Relative activity[2]
Unheated Control (0 ºC)	2.8	100%
25 ºC	3.1	110%
37 ºC	2.5	88%
55 ºC	0.70	25%
75 ºC	< LOD[3]	< 8%[4]
95 ºC	< LOD[3]	< 8%[4]

[1] Mean specific activity determined from n = 2 for each treated sample (one at each temperature and time point),.
[2]CP4 EPSPS activity of unheated control was assigned 100 %.
[3] LOD is defined as the value that is three standard deviations above the mean of the assay blank.
[4] Calculated from the LOD of the CP4 EPSPS activity assay.

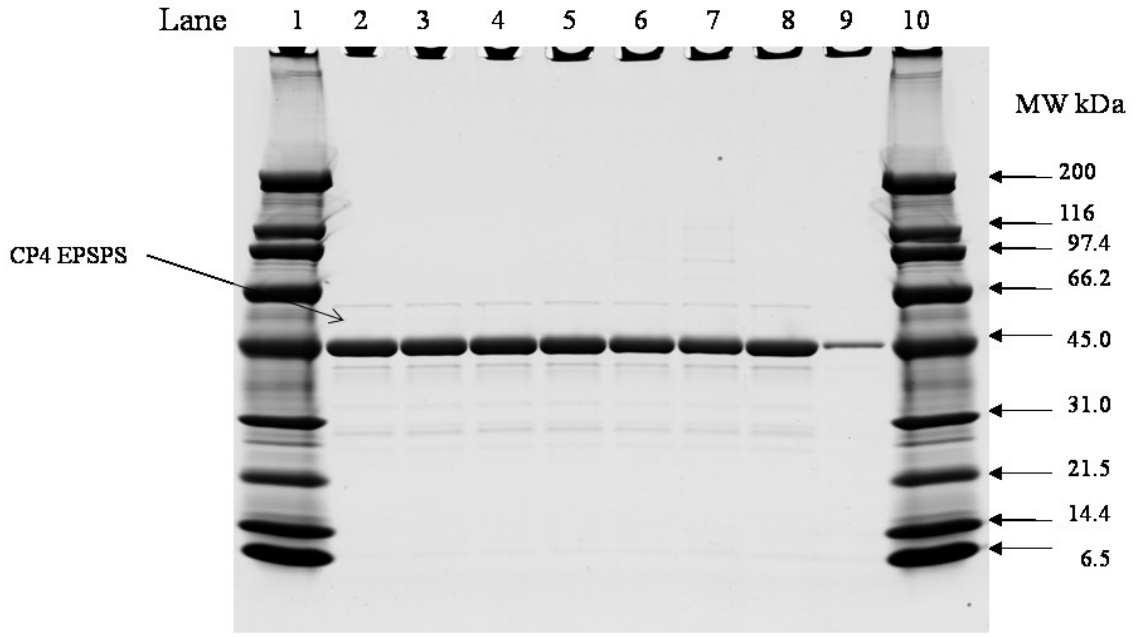

Figure 34 SDS-PAGE of CP4 EPSPS Following Heat Treatment for 15 Minutes

Heated-treated samples of CP4 EPSPS (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

Lane	Description	Amount (µg)
1	Broad Range Molecular Weight Markers	4.5
2	CP4 EPSPS Temperature Unheated Control (0 ºC)	3.2
3	CP4 EPSPS 25 ºC	3.2
4	CP4 EPSPS 37 ºC	3.2
5	CP4 EPSPS 55 ºC	3.2
6	CP4 EPSPS 75 ºC	3.2
7	CP4 EPSPS 95 ºC	3.2
8	CP4 EPSPS Reference	3.2
9	CP4 EPSPS Reference	0.32
10	Broad Range Molecular Weight Markers	4.5

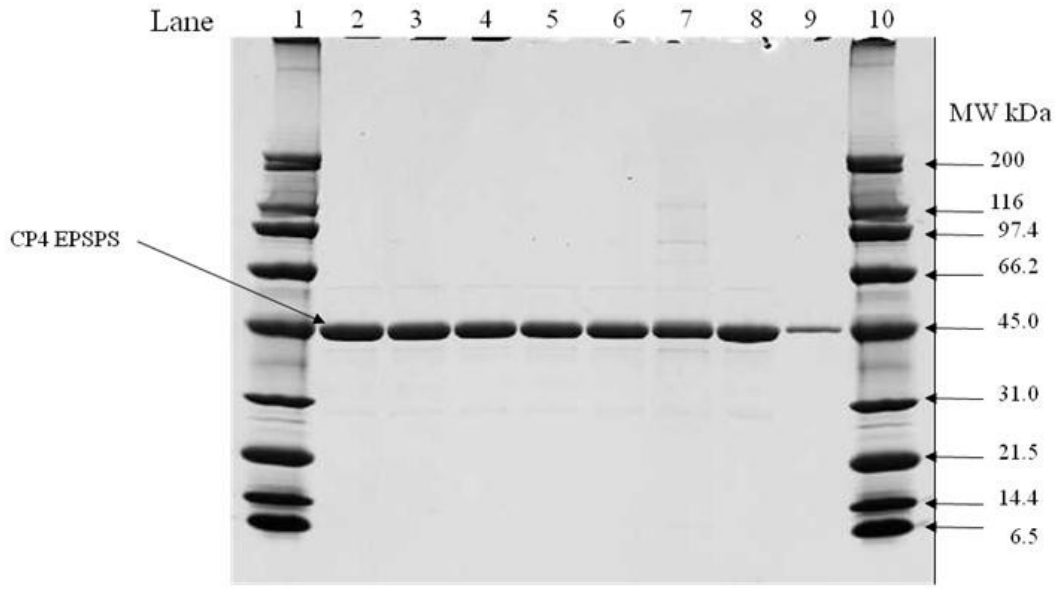

Figure 35 SDS-PAGE of CP4 EPSPS Following Heat Treatment for 30 Minutes

Heated samples of CP4 EPSPS protein (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

Lane	Description	Amount (µg)
1	Broad Range Molecular Weight Markers	4.5
2	CP4 EPSPS 25 ºC	3.2
3	CP4 EPSPS 37 ºC	3.2
4	CP4 EPSPS 55 ºC	3.2
5	CP4 EPSPS 75 ºC	3.2
6	CP4 EPSPS 95 ºC	3.2
7	CP4 EPSPS Unheated Control (0 ºC)	3.2
8	CP4 EPSPS Reference	3.2
9	CP4 EPSPS Reference	0.32
10	Broad Range Molecular Weight Markers	4.5

B4(c) Acute or short-term oral toxicity on novel protein(s)

Most known protein toxins act through acute mechanisms to exert toxicity (Hammond and Fuchs, 1998; Pariza and Johnson, 2001; Sjoblad *et al.*, 1992). The primary exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which manifest toxicity in a short term (few weeks) feeding study (Liener, 1994). The amino acid sequence of the Cry3Bb1 and CP4 EPSPS proteins produced in MON 87411 are not similar to any of these anti-nutritional proteins or to any other known protein toxin. Therefore, an acute oral mouse toxicity study was considered sufficient to evaluate the toxicity of the Cry3Bb1 and CP4 EPSPS protein.

E. coli-produced Cry3Bb1 protein was administered by gavage to 10 male and 10 female CD-1 mice at dose of 1930 mg/kg body wt (bw). Additionally, 10 male and 10 female mice were administered a comparable amount (mg/kg bw) of bovine serum albumin (BSA) to serve as a protein controls, respectively. Following dosing, all mice were observed daily for mortality or signs of toxicity. Food consumption was measured weekly. Body weights were measured prior to dosing and at study days 7 and 14. All animals were sacrificed on day 14 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight gain, food consumption or gross pathology. Therefore, the No Observable Adverse Effect Level (NOAEL) for Cry3Bb1was considered to be 1930 mg/kg bw.

E. coli-produced CP4 EPSPS protein was administered as a single dose by gavage to three groups of 10 male and 10 female CD-1 mice at dose levels up to 572 mg/kg body wt (bw) (Harrison *et al.*, 1996). Additional groups of mice were administered comparable volume of the buffer or a comparable amount (mg/kg bw) of bovine serum albumin (BSA) to serve as vehicle or protein controls, respectively. Following dosing, all mice were observed twice daily for mortality or signs of toxicity. Food consumption was measured daily. Body weights were measured prior to dosing and at study day 7. All animals were sacrificed on day 8 or 9 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight gain, food consumption or gross pathology. Therefore, the No Observable Adverse Effect Level (NOAEL) for CP4 EPSPS was considered to be 572 mg/kg bw.

For details please refer to Kaempfe and Bonner, 2003 (MSL0018711) and Naylor, 1993 (MSL0013077).

B5 Assessment of Potential Allergenicity

Please see Section B4 for assessment of DvSnf7.

History of safe use of the introduced protein is a key consideration in the potential for allergenicity and toxicity and for assessment of dietary safety. The history of safe use of Cry3Bb1 and CP4 EPSPS proteins have been previously addressed in Section B3(f).

Following the guidelines adopted by the Codex Alimentarius Commission, an assessment of potential allergenicity of introduced proteins has been conducted, by comparing the characteristics of the introduced protein to characteristics of known allergens (Codex Alimentarius, 2009). A protein is not likely to be associated with allergenicity if: 1) the protein is from a nonallergenic source; 2) the protein represents only a very small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence; 4) the protein does not show resistance to pepsin digestion; and 5) the protein is not stable to heat treatment. The Cry3Bb1 and CP4 EPSPS proteins in MON 87411 have been assessed for their potential allergenicity according to these safety assessment guidelines .

B5(a) Source of introduced protein

B5(a)(i) Safety of the donor organism of *Bacillus thuringiensis*

The donor organism for *cry3Bb1*, *Bacillus thuringiensis,* has been used commercially in the United States since 1958 to produce microbial-derived products with insecticidal activity. The extremely low mammalian toxicity of *B.t.*-based insecticide products has been demonstrated in numerous safety studies, and there are no confirmed cases of allergic reactions to Cry proteins in applicators of microbial-derived *B.t.* products during 50 years of use.

Applications of sporulated *B.t* have a long history of safe use for pest control in agriculture, especially in organic farming (Cannon, 1993; WHO, 1999; U.S. EPA, 1988). Microbial pesticides containing *B.t.* Cry3 proteins have been used for more than 30 years and subjected to extensive toxicity testing showing no adverse effects to human health (Baum *et al.*, 1999; Betz *et al.*, 2000; McClintock *et al.*, 1995; Mendelsohn *et al.*, 2003; U.S. EPA, 2001a; 2005).

B5(a)(ii) Safety of the donor organism of *Agrobacterium* sp. strain CP4

The donor organism, *Agrobacterium* sp. strain CP4, was isolated based on its tolerance to glyphosate brought about by the production of a naturally glyphosate-tolerant EPSPS protein (Padgette *et al.*, 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are not commonly known for human or animal pathogenicity or allergenicity. According to a report of a joint FAO/WHO Expert Consultation (Helm, 2001), there is no known population of individuals sensitized to bacterial proteins. Furthermore, *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with various regulatory agencies worldwide regarding Roundup Ready soybean (40-3-2), Roundup Ready 2 Yield soybean (MON 89788), Roundup Ready corn 2 (NK603), Roundup Ready canola (RT73), Roundup Ready sugar beet (H7-1),

Roundup Ready cotton (MON 1445), Roundup Ready Flex cotton (MON 88913) and Roundup Ready alfalfa (J101 x J163).

B5(b) Bioinformatic comparison (aa) of novel protein(s) to allergens

B5(b)(i) Structural similarity of Cry3Bb1 to known allergens

The Codex guidelines for the evaluation of the allergenicity potential of introduced proteins (Codex Alimentarius, 2009) are based on the comparison of amino acid sequences between introduced proteins and allergens, where allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with an allergen over any segment of at least 80 amino acids. The Codex guideline also recommends that a sliding window search with a scientifically justified peptide size could be used to identify immunologically relevant peptides in otherwise unrelated proteins. Therefore, the extent of sequence similarities between the Cry3Bb1 protein sequence and known allergens, gliadins, and glutenins was assessed using the FASTA sequence alignment tool and an eight-amino acid sliding window search (Codex Alimentarius, 2009; Thomas *et al.*, 2005). The data generated from these analyses confirm that the Cry3Bb1 protein does not share amino acid sequence similarities with known allergens, gliadins, or glutenins.

The FASTA program directly compares amino acid sequences (i.e. primary, linear protein structure). This alignment data may be used to infer shared higher order structural similarities between two sequences (i.e. secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. By definition, homologous proteins have common secondary structures, and three-dimensional configuration, and, consequently, may share similar functions. The allergen, gliadin, and glutenin sequence database (AD_2013) was obtained from Food Allergy Research and Resource Program Database (FARRP, 2011) and was used for the evaluation of sequence similarities shared between the Cry3Bb1 protein and all proteins. The AD_2013 database contains 1,630 sequences. When used to align the sequence of the introduced protein to each protein in the database, the FASTA algorithm produces an *E*-score (expectation score) for each alignment. The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a low degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences which have an *E*-score of less than or equal to 1×10^{-5} are considered to have meaningful homology. Results indicate that the Cry3Bb1 protein sequence does not share meaningful similarity with sequences in the allergen database. No alignment met nor exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2009) or had an *E*-score of less than or equal to 1×10^{-5}.

A second bioinformatic tool, an eight-amino acid sliding window search, was used to specifically identify short linear polypeptide matches to known allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may contain smaller immunologically meaningful epitopes. An amino acid sequence may have allergenic potential if it has an exact sequence identity of at least eight linearly contiguous amino acids with a potential allergen epitope (Hileman *et al.*, 2002; Metcalfe *et al.*, 1996). Using a

sliding window of less than eight amino acids can produce matches containing considerable uncertainty depending on the length of the query sequence (Silvanovich *et al.*, 2006) and are not useful to the allergy assessment process (Thomas *et al.*, 2005). No eight contiguous amino acid identities were detected when the Cry3Bb1 protein sequence was compared to the proteins in the AD_2013 sequence database.

The bioinformatic results demonstrated there were no biologically relevant sequence similarities to allergens when the Cry3Bb1 protein sequence was used as a query for a FASTA search of the AD_2013 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the Cry3Bb1 protein sequence and proteins in the allergen database. These data show that the Cry3Bb1 protein sequence lacks both structurally and immunologically relevant similarities to known allergens, gliadins, and glutenins.

B5(b)(ii) Structural similarity of CP4 EPSPS protein to known allergens

The structural similarity of CP4 EPSPS protein to known allergens was assessed following the same Codex guidelines summarized above for Cry3Bb1 (Section B5(b)(i)) data generated from these analyses confirm that the CP4 EPSPS protein does not share amino acid sequence similarities with known allergens, gliadins, or glutenins.

Results indicate that the CP4 EPSPS protein sequence does not share significant similarity with sequences in the allergen database. No alignment met nor exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2009) or had an *E*-score of less than or equal to 1×10^{-5}.

Additionally, no eight contiguous amino acid identities were detected when the CP4 EPSPS protein sequence was compared to the proteins in the AD_2013 sequence database.

Results show there were no similarities to allergens when the CP4 EPSPS protein sequence was used as a query for a FASTA search of the AD_2013 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the CP4 EPSPS protein sequence and proteins in the allergen database. These data show that the CP4 EPSPS protein sequence lacks both structurally and immunologically relevant sequence similarities to known allergens, gliadins, and glutenins.

B5(c) Structural properties, including digestion by pepsin, heat treatment

B5(c)(i) Digestibility of proteins

Proteins introduced into commercial food crops using biotechnology are evaluated for their safety for human and animal consumption. One aspect of this assessment is an evaluation of a protein's susceptibility to proteolytic digestion with enzymes of the gastrointestinal tract. Certain proteins can withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood *et al.*, 1996; Moreno *et al.*, 2005; Vassilopoulou *et al.*, 2006; Vieths *et al.*, 1999). A correlation between protein digestibility with pepsin in simulated gastric fluid (SGF) and the likelihood of the protein being an allergen has been previously reported (Astwood *et al.*, 1996), although this correlation is not complete (Fu *et al.*, 2002). The SGF assay serves as a tool to compare the relative susceptibility of proteins to digestion

in pepsin. The SGF assay protocol has been standardized based on results obtained from an international, multi-laboratory study (Thomas *et al.*, 2004). This study showed that the standardized protocol provides reproducibility and consistency for determining the digestive stability of a protein. Using this standardized protocol, the digestive stability of Cry3Bb1 protein was analyzed.

Simulated intestinal fluid (SIF) has also been used as a stand-alone independent test system to assess the digestibility of food components (Okunuki *et al.*, 2002). The relationship between protein allergenicity and protein stability in the *in vitro* stand-alone SIF study is limited, because the protein has not been first exposed to the acidic, denaturing conditions simulating the stomach, as would be the case with *in vivo* digestion (FAO-WHO, 2001). Nonetheless degradation of protein in SIF can be evaluated to better understand the digestive fate of a protein and hence, the digestive stability of Cry3Bb1 protein in SIF was also assessed.

B5(c)(i)1 Digestive fate of the Cry3Bb1 protein

The digestibility of *E. coli*-produced Cry3Bb1 protein in SGF was assessed using two methods: analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel and visual analysis of a western blot probed with a goat anti-Cry3Bb1 polyclonal antibody. Digestibility of Cry3Bb1 protein in SGF was evaluated over time by analyzing digestion mixtures incubated for targeted time intervals. For SDS-PAGE analysis, approximately 0.5 µg of Cry3Bb1 protein was analyzed for each time point (Figure 36). The no protein control at digestion time 0 and no protein control at digestion time 60 (Figure 36, Lanes 12 and 24), which evaluate the stability of the pepsin in the test system (SGF) lacking the Cry3Bb1 protein, demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase. In addition, no change in the Cry3Bb1 protein band intensity was observed over time in the absence of pepsin (compare no pepsin control at digestion time 0 sec to no pepsin control at digestion time 60 min; Figure 36, Lanes 13 and 25) indicating that the digestion of the Cry3Bb1 protein was due to the proteolytic activity of pepsin present in SGF and not due to instability of the protein while incubated at pH ~1.2 at ~37 °C for 60 min.

SDS-PAGE data showed that the intact Cry3Bb1 protein was completely digested within 15 sec of incubation in SGF (Figure 36, Lane 15). A protein fragment (migrating near the dye front) was observed in the first five time points. This low molecular weight protein fragment was completely digested to a level below the limit of detection after four minutes in SGF (Figure 36, Lanes 20-24). For the SDS-PAGE analysis, the LOD of the Cry3Bb1 protein was visually estimated to be 10 ng, or 0.010 µg (Figure 36, Lane 6). This LOD used to calculate the maximum amount of intact Cry3Bb1 protein that could remain visually undetected after digestion, which corresponded to approximately 2.0% of the Cry3Bb1 protein loaded. Based on that LOD, more than 98.0% (100.0% - 2.0% = 98.0%) of the intact Cry3Bb1 protein was digested within 15 sec of incubation in SGF.

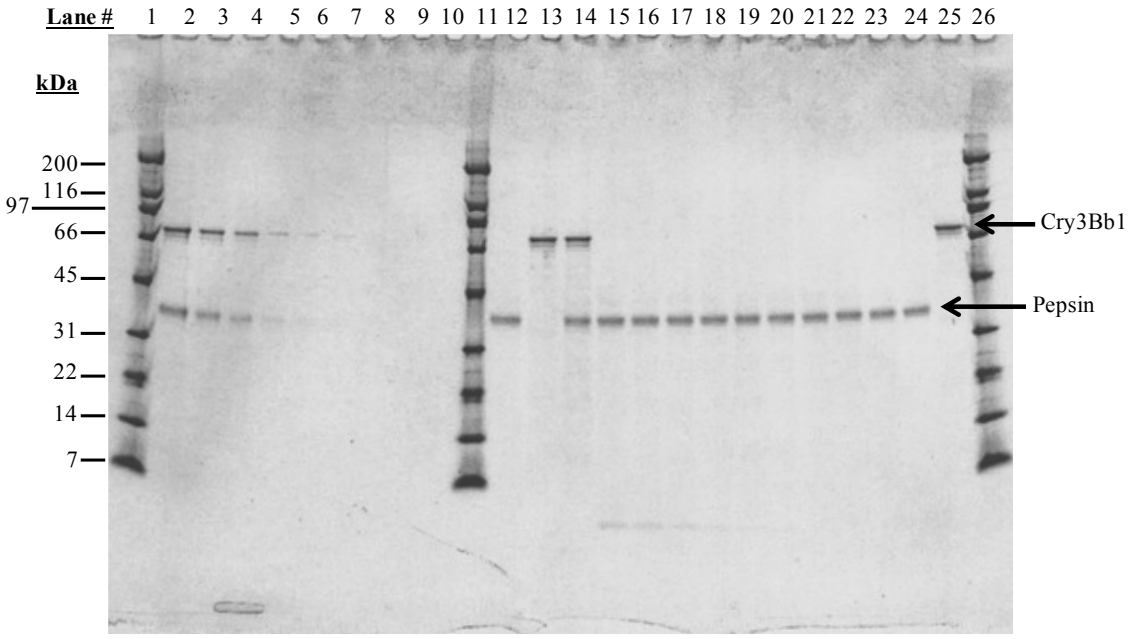

Figure 36 Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-produced Cry3Bb1Protein in Simulated Gastric Fluid

Proteins were subjected to SDS-PAGE and detected by staining with Brilliant Blue G Colloidal stain. Approximate molecular weights are shown on the left of each gel, and correspond to the markers (MWM) loaded. Lanes 2 through 10 corresponds to the analysis to determine LOD of Cry3Bb1 protein. The amount of *E. coli*-produced Cry3Bb1 protein in the SGF0 sample loaded to estimate the LOD are indicated below. Lanes 12 through 25 corresponds to Cry3Bb1 protein digestion in SGF. *E. coli*-produced Cry3Bb1protein was loaded at 0.5 µg per lane based on pre-digestion concentrations. Lane designations are as follows:

Lane	Sample	Amount (ng)	Lane	Sample	Digestion Time
1	MWM		12	No Cry3Bb control	0 sec
2	SGF0	500	13	No pepsin control	0 sec
3	SGF0	200	14	SGF0	0 sec
4	SGF0	100	15	SGF1	15 sec
5	SGF0	50	16	SGF2	30 sec
6	SGF0	25	17	SGF3	1 min
7	SGF0	10	18	SGF4	2 min
8	SGF0	5	19	SGF5	4 min
9	SGF0	2.5	20	SGF6	8 min
10	SGF0	1	21	SGF7	15 min
11	MWM		22	SGF8	30 min
			23	SGF9	60 min
			24	No Cry3Bb1 control	60 min
			25	No pepsin control	60 min
			26	MWM	

The digestibility of the Cry3Bbl protein in SGF was also evaluated using western blot analysis. The Cry3Bbl protein was loaded at 10 ng per lane (based on pre-digestion concentration and purity values). Examination of the SGF western blot (Figure 37) confirmed the results of the Colloidal Brilliant Blue G stained polyacrylamide gel. The full-length Cry3Bb1 protein (~77 kDa) was digested below the LOD within 15 sec of incubation in SGF (Figure 37, Lane 15). No proteolytic fragments were observed for any of the time points. No bands were observed when Cry3Bb1 protein was absent (Figure 37, Lanes 12 and 24, respectively), demonstrating that the immunoreactive signal observed on this blot was due solely to Cry3Bbl protein. No change in the Cry3Bb1 protein band intensity was observed in the no pepsin controls (Figure 37, Lanes 13 and 25). This result reaffirms that the Cry3Bb1 protein was stable in the assay system without pepsin and indicates that degradation of the Cry3Bbl protein was due to digestion by pepsin.

The LOD of the Cry3Bb1 protein in the western blot analysis was estimated to be 0.02 ng (Figure 37, Lane 9). The LOD estimated was used to calculate the maximum amount of intact CryBb1 protein that could remain visually undetected after digestion. This corresponded to approximately 0.2% of the total Cry3Bb1 protein loaded. Based on the western blot LOD, it can be concluded that within 15 sec more than 99% (100% - 0.2% = 99.8%) of the intact Cry3Bb1 protein was digested.

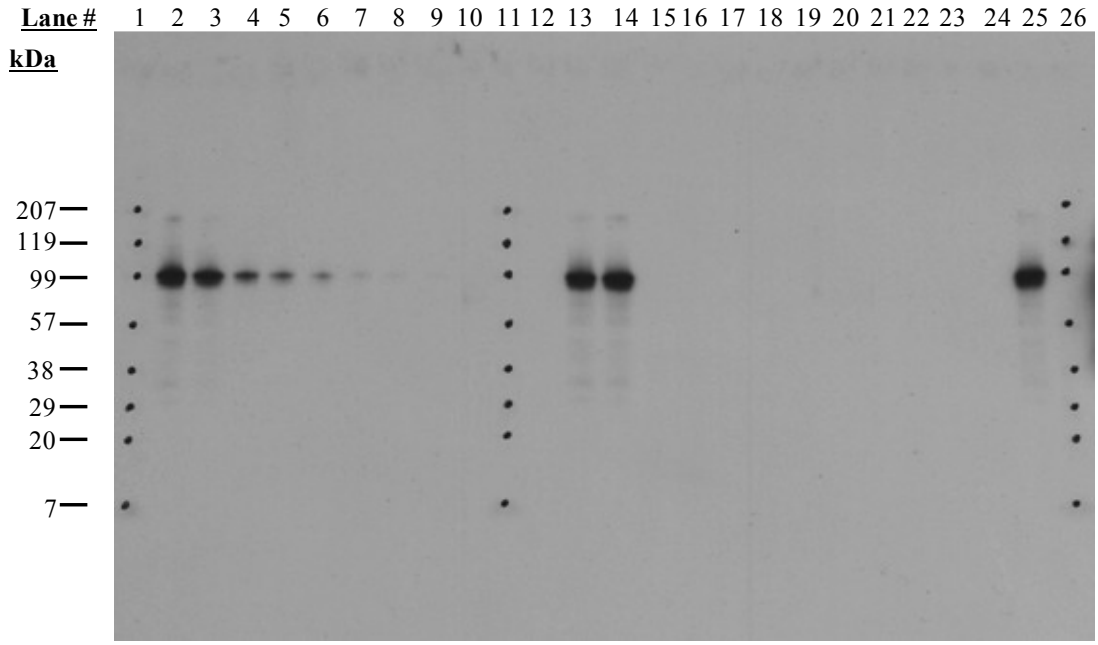

Figure 37 Western Blot Analysis of Purified *E. coli*-produced Cry3Bb1 Protein in Simulated Gastric Fluid

Western blots probed with an anti-Cry3Bb1 antibody were used to assess the digestibility of Cry3Bb1 protein in SGF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. A 5 sec exposure is shown. Lanes 12 through 25 corresponds to *E. coli*-produced Cry3Bb1 protein digestion in SGF. Based on pre-digestion protein concentration, 10 ng of Cry3Bb1 protein was loaded in each lane containing Cry3Bb1 protein (No pepsin control, SGF0-SGF9). Lanes 2 through 10 corresponds to the analysis to determine LOD of *E. coli*-produced Cry3Bb1 protein. Indicated amounts of the Cry3Bb1 protein from the SGF0 sample were loaded to estimate the LOD of the protein. Lane designations are as follows:

Lane	Sample	Amount (ng)	Lane	Sample	Digestion Time
1	MWM		12	No Cry3Bb control	0 sec
2	SGF0	10	13	No pepsin control	0 sec
3	SGF0	5	14	SGF0	0 sec
4	SGF0	1	15	SGF1	15 sec
5	SGF0	0.5	16	SGF2	30 sec
6	SGF0	0.2	17	SGF3	1 min
7	SGF0	0.1	18	SGF4	2 min
8	SGF0	0.05	19	SGF5	4 min
9	SGF0	0.02	20	SGF6	8 min
10	SGF0	0.01	21	SGF7	15 min
11	MWM		22	SGF8	30 min
			23	SGF9	60 min
			24	No Cry3Bb1 control	60 min
			25	No pepsin control	60 min
			26	MWM	

The digestibility of the *E. coli*-produced Cry3Bb1 protein in SIF was assessed by western blot (Figure 38). Methods for SIF analysis are described in Appendix F. The gel used to assess the digestibility of the Cry3Bb1 protein in SIF by western blot was loaded with 10 ng of the Cry3Bbl protein (based on pre-digestion concentration and purity values) for each of the incubation time points. No immunoreactive bands were observed in the no protein controls, which represent the SIF test system without Cry3Bb1 protein (Figure 38, Lanes 11 and 24). This result demonstrates the absence of non-specific antibody interactions with the SIF test system.

Western blot analysis indicated that the full-length Cry3Bbl protein (~77 kDa) was digested within 1 minute in SIF (Figure 38, Lane 14). As expected, stable proteolytic fragments of the Cry3Bbl protein were present throughout the 24 hour incubation in SIF. At the 1 minute time point, two major proteolytic fragments were observed with molecular weights of 70 and 66 kDa (Figure 38, Lane 14). As the incubation time in SIF increased, the 70 kDa proteolytic fragment decreased in intensity while the ~66 kDa fragment remained at about the same intensity. At the 1 minute time point, other proteolytic fragments ranging from 30-60 kDa in molecular weight also began to accumulate (Figure 38, Lane 14). The intensity of these lower molecular weight fragments increased as the incubation time in SIF increased, indicating degradation of the Cry3Bbl protein. A faint protein band, consistent with full-length Cry3Bbl protein, was observed in lane 22 but not in lanes 14-21 or lane 23 of the SIF Western blot (Figure 38). This anomaly is probably due to aggregation of protein and was unique to the 12 hour incubation time point.

Overall, this digestion pattern was expected for the Cry3Bb1 protein. Pancreatin contains trypsin and other proteases, which are known to digest Cry proteins . Proteolytic conversion of the full-length protoxin to an active toxin has been described for all members of the Cryl, Cry2, Cry3 and Cry4 class proteins (Rukmini *et al.*, 2000). When exposed to trypsin or trypsin-like insect midgut proteases, Cry proteins are degraded to a stable "tryptic core" (Höfte and Whiteley, 1989). No bands were observed when Cry3Bbl protein was absent (Figure 38, Lanes 11 and 24, respectively), demonstrating that the immunoreactive signal observed on this blot was due solely to Cry3Bb1 protein. No pancreatin controls that contained the Cry3Bbl protein but lacked pancreatin were loaded in lanes 12 and 25. No change in the Cry3Bbl band intensities was observed when exposed to SIF without pancreatin for 24 hours. This demonstrated that degradation of the Cry3Bbl protein was due to digestion by pancreatin and not instability of the test substance at pH 7.5 and incubation at 37 °C.

To determine the LOD for Cry3Bbl protein in SIF by western blot analysis, dilutions of the SIF0 sample (loaded based on pre-digestion concentration and purity values) were analyzed on the same western blot (Figure 38, Lanes 2-9). The lowest amount of Cry3Bbl protein observed on the Western blot was 0.05 ng (Figure 38, Lane 8). Therefore the LOD for Cry3Bbl protein was established to be 0.05 ng. Based on the LOD, it was determined that the full-length protein was at least 99.5% digested within 1 minute of incubation in SIF.

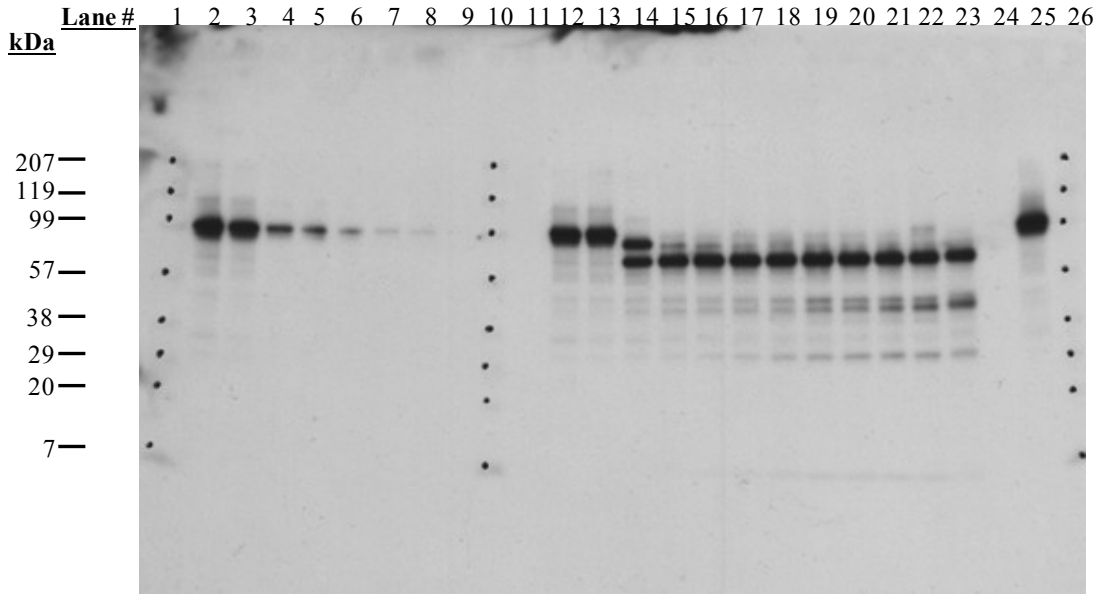

Figure 38 Western Blot Analysis of Purified *E. coli*-produced Cry3Bb1 Protein in Simulated Intestinal Fluid

Western blots probed with an anti-Cry3Bb1 antibody were used to assess the digestibility of Cry3Bb1 protein in SIF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. A 10 sec exposure is shown. Lanes 11 through 25 corresponds to *E. coli*-produced Cry3Bb1 protein digestion in SIF. Based on pre-digestion protein concentration, 10 ng of the Cry3Bb1 protein was loaded in each lane containing Cry3Bb1 protein. Lanes 2 through 9 corresponds to the analysis to determine LOD of *E. coli*-produced Cry3Bb1 protein. Indicated amounts of the Cry3Bb1 protein from the SIF0 sample were loaded to estimate the LOD of the protein. Lane designations are as follows:

Lane	Sample	Amount (ng)	Lane	Sample	Digestion Time
1	MWM		11	No Cry3Bb control	0 min
2	SIF0	10	12	No panceatin control	0 min
3	SIF0	5	13	SIF0	0 min
4	SIF0	1	14	SIF1	1 min
5	SIF0	0.5	15	SIF2	5 min
6	SIF0	0.2	16	SIF3	15 min
7	SIF0	0.1	17	SIF4	30 min
8	SIF0	0.05	18	SIF5	1 hr
9	SIF0	0.01	19	SIF6	2 hr
10	MWM		20	SIF7	4 hr
			21	SIF8	6 hr
			22	SIF9	12 hr
			23	SIF10	24 hr
			24	No Cry3Bb1 control	24 hr
			25	No pancreatin control	24 hr
			26	MWM	

Digestive fate of the Cry3Bb1 protein - Conclusions

Experiments designed to test the digestibility of the Cry3Bbl protein in simulated digestive fluids were performed. Results indicate that the Cry3Bb1 protein was rapidly digested when incubated in SGF. At least 98% of the full length (~77 kDa) Cry3Bbl protein was digested within 15 seconds in SGF when analyzed using Colloidal Brilliant Blue G stained SDS polyacrylamide gels. A proteolytic fragment (migrating at the dye front) was observed in the initial time points, but was absent after four minutes of incubation in SGF. At least 99.8% of the full length Cry3Bb1 protein was digested within 15 seconds in SGF when evaluated using western blot analysis. No proteolytic fragments were observed for samples evaluated using western blot analysis. The full length Cry3Bb1 protein was also rapidly digested when incubated in SIF. At least 99.5% of the full length Cry3Bbl protein was digested within 1 minute in SIF when evaluated using western blot analysis. As expected, protease resistant fragments were observed throughout the 24 hour time course of the SIF digestion, since proteolytic (trypsin) conversion of the full length protoxin to an active toxin has been described for all members of the Cryl, Cry2, Cry3, and Cry4 families. Taken together, these results suggest that Cry3Bb 1 protein will be readily digestible in the mammalian digestive tract.

For details please refer to Bonner *et al.*, 2003 (MSL0018662)

B5(c)(i)2 Digestive fate of the CP4 EPSPS protein

Harrison *et al*. (1996) demonstrated that the *E. coli*-produced CP4 EPSPS protein is rapidly degraded in simulated digestive fluids. Based on Western blot analysis, CP4 EPSPS protein was undetectable within 15 seconds under simulated gastric conditions greatly minimizing the potential for this protein to be absorbed in the intestinal mucosa. In addition, when digested in simulated intestinal fluid (SIF), the half life of CP4 EPSPS protein was less than 10 minutes (Harrison *et al.*, 1996). Therefore, if any of the CP4 EPSPS protein were to survive in the gastric system, it is expected that it would be rapidly degraded in the intestine. Based on this information, CP4 EPSPS protein is expected to degrade rapidly in the mammalian digestive tract.

Subsequent experiments using the standardized method published by the International Life Science Institute (ILSI) (Thomas *et al.*, 2004), confirmed the *in vitro* digestibility of the *E. coli*-produced CP4 EPSPS protein in SGF. *E. coli*-produced CP4 EPSPS protein, shown to be physiochemically and functionally equivalent to the CP4 EPSPS protein produced in MON 87411 (Section B1(b)), was used in these experiments. Similar to the results reported by Harrison *et al*. (1996), greater than 98% of the CP4 EPSPS protein was digested within 15 sec, based on the results of visual inspection of colloidal blue stained SDS-PAGE gels (Figure 39). Western blot analysis confirmed that greater than 95% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 sec (Figure 40). In summary, the results of these experiments confirmed that the *E. coli*-produced CP4 EPSPS protein was rapidly digested after incubation in SGF and is therefore unlikely to pose a human health concern.

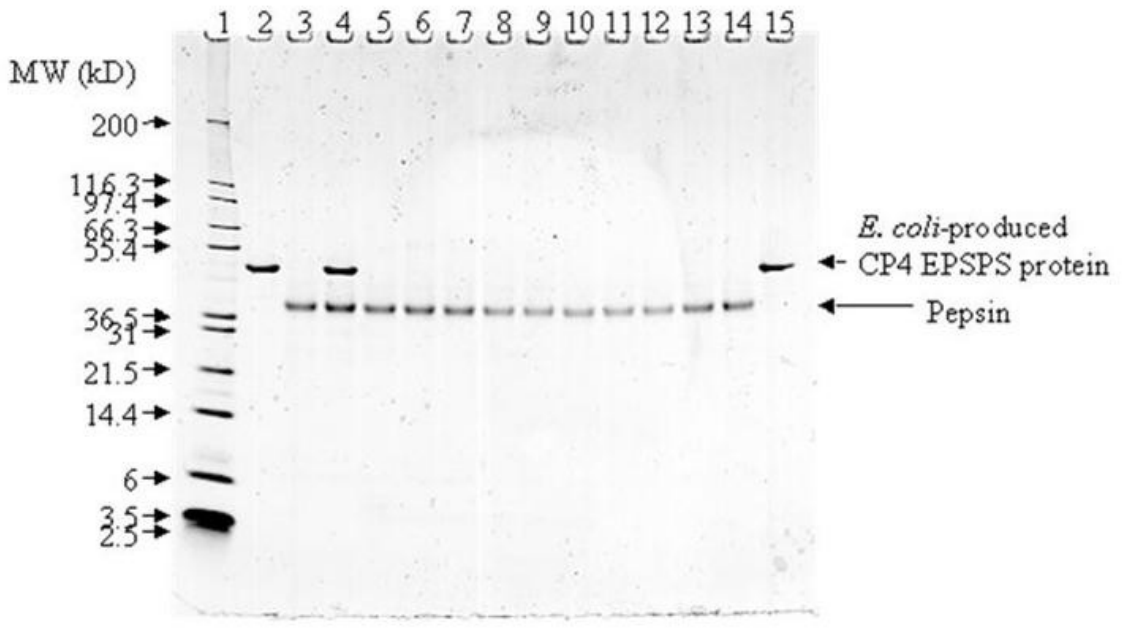

Figure 39 Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid

Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G Colloidal stain. *E. coli*-produced CP4 EPSPS protein was loaded at 500 ng per lane based on pre-digestion concentrations.

Lane	Description	Incubation Time
1	Molecular weight markers (Invitrogen P/N LC 5677)	
2	Experimental control without pepsin	0
3	Experimental control without CP4 EPSPS	0
4	CP4 EPSPS protein in SGF	0
5	CP4 EPSPS protein in SGF	15 sec
6	CP4 EPSPS protein in SGF	30 sec
7	CP4 EPSPS protein in SGF	1 min
8	CP4 EPSPS protein in SGF	2 min
9	CP4 EPSPS protein in SGF	4 min
10	CP4 EPSPS protein in SGF	8 min
11	CP4 EPSPS protein in SGF	15 min
12	CP4 EPSPS protein in SGF	30 min
13	CP4 EPSPS protein in SGF	60 min
14	Experimental control without CP4 EPSPS	60 min
15	Experimental control without pepsin	60 min

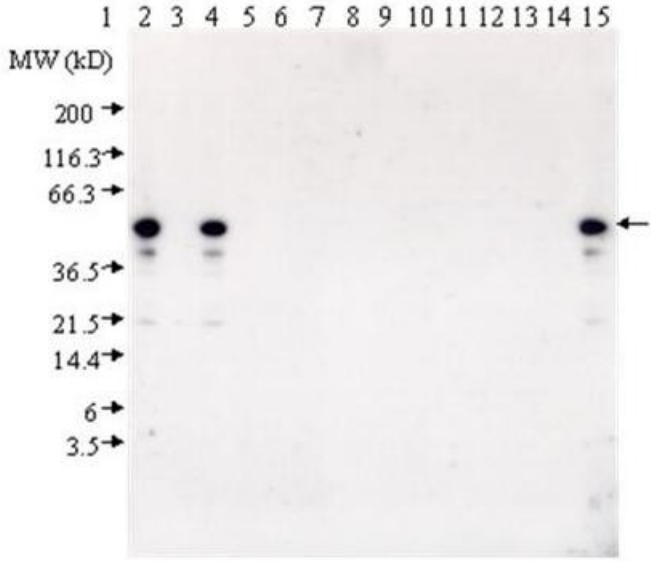

Figure 40 Western Blot Analysis of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid

Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a tricine buffered gel, electroblotted, and probed with anti-CP4 EPSPS goat serum. *E. coli*-produced CP4 EPSPS protein was loaded at 1 ng per lane based on 90% purity and pre-digestion concentrations. Lane 1 containing the molecular weight markers (Invitrogen P/N LC5677) was cropped and the arrow on the right side of the image indicates the band corresponding to CP4 EPSPS protein.

Lane	Description	Incubation Time
1	Molecular weight markers	
2	Experimental control without pepsin	0
3	Experimental control without CP4 EPSPS	0
4	CP4 EPSPS protein in SGF	0
5	CP4 EPSPS protein in SGF	15 sec
6	CP4 EPSPS protein in SGF	30 sec
7	CP4 EPSPS protein in SGF	1 min
8	CP4 EPSPS protein in SGF	2 min
9	CP4 EPSPS protein in SGF	4 min
10	CP4 EPSPS protein in SGF	8 min
11	CP4 EPSPS protein in SGF	15 min
12	CP4 EPSPS protein in SGF	30 min
13	CP4 EPSPS protein in SGF	60 min
14	Experimental control without CP4 EPSPS	60 min
15	Experimental control without pepsin	60 min

E. coli-produced CP4 EPSPS degradation in simulated intestinal fluid (SIF) was assessed by western blot analysis. Greater than 50% of the CP4 EPSPS was degraded after a 10 min incubation in SIF at 37°C (Figure 41, Lane 6) compared to the level detected at time zero (Lane 5). No CP4 EPSPS protein was detected after incubation in SIF for 100 min or longer (Lanes 8-10 and 12).

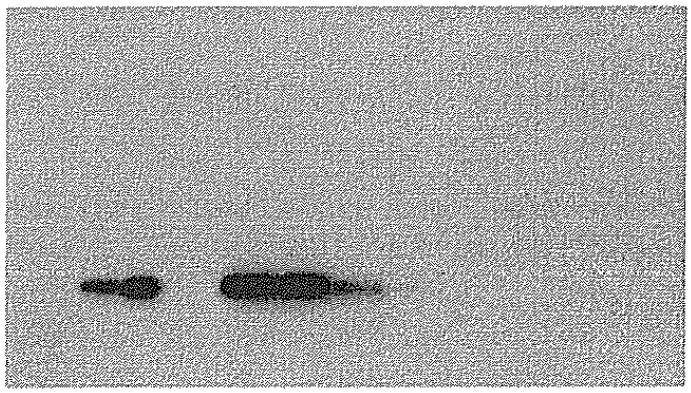

Figure 41 Western Blot Analysis of CP4 EPSPS Degradation in Simulated Intestinal Fluid

CP4 EPSPS was added to SIF to a final concentration of 50 μg/ml, and incubated at 37°C for the designated duration as indicated below. The reactions were terminated by heating at ~100°C for 5 min before analysis by SDS-PAGE followed by western.

Lane	Sample	Time of incubation (min)
1	CP4 EPSPS (5 ng)	0
2	CP4 EPSPS (10 ng)	0
3	SIF only	0
4	CP4 EPSPS (10 ng)	0
5	CP4 EPSPS (10 ng) + SIF	0
6	CP4 EPSPS (10 ng) + SIF	10
7	CP4 EPSPS (10 ng) + SIF	32
8	CP4 EPSPS (10 ng) + SIF	100
9	CP4 EPSPS (10 ng) + SIF	270
10	CP4 EPSPS (10 ng) + SIF	1181
11	SIF only	1171
12	CP4 EPSPS (10 ng) + SIF	1160

Digestive fate of CP4 EPSPS protein - Conclusions

Experiments designed to test the digestibility of the CP4 EPSPS protein in simulated digestive fluids were performed. Results indicate that the CP4 EPSPS protein was rapidly digested when incubated in SGF. At least 98% of the full length CP4 EPSPS protein was digested within 15 seconds in SGF when analyzed using Colloidal Brilliant Blue G stained SDS polyacrylamide gels. At least 95% of the CP4 EPSPS protein was digested within 15 seconds in SGF when evaluated using western blot analysis. No proteolytic fragments were observed for samples evaluated using western blot analysis. The CP4 EPSPS protein was also rapidly digested when incubated in SIF. Greater than 50% of the CP4 EPSPS protein was digested within 10 minute in SIF when evaluated using western blot analysis. Taken together, these results indicate that the CP4 EPSPS protein will be readily digestible in the mammalian digestive tract.

For details please refer to Leach *et al.*, 2002 (MSL0017566).

B5(c)(ii) Heat stability of proteins

B5(c)(ii)1 Heat stability of the Cry3Bb1 protein

Exposure to heat during food processing or cooking is likely to have a profound effect on the structure and function of proteins. Therefore the effect of heat treatment on the activity of Cry3Bb1 protein was evaluated using functional assays to assess the impact of temperature on functional activity, and SDS-PAGE to assess the impact on protein integrity. The results show that Cry3Bb1 protein was completely inactivated by heating to temperatures above 75°C. These data are presented and discussed in more detail in Section B4(b)(ii).

B5(c)(ii)2 Heat stability of the CP4 EPSPS protein

Exposure to heat during food processing or cooking is likely to have a profound effect on the structure and function of proteins. Therefore the effect of heat treatment on the activity of CP4 EPSPS protein was evaluated using functional assays to assess the impact of temperature on functional activity, and SDS-PAGE to assess the impact on protein integrity. The results show that CP4EPSPS protein was completely inactivated by heating to temperatures above 75°C. These data are presented and discussed in more detail in Section B4(b)(ii).

B5(d) Specific serum screening if protein from allergenic source

Not applicable.

B5(e) Protein as a proportion of total protein

B5(e)(i) The Cry3Bb1 protein as a proportion of total protein

The Cry3Bb1 protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table 21). Among tested tissues of MON 87411, grain is the most relevant to the assessment of food allergenicity since grain is a food and feed source. The mean level of Cry3Bb1 protein in grain of MON 87411 is 4.0 µg/g dw. The mean percent dry weight of total protein in grain of MON 87411 is 10.71% (or 107,100 µg/g). The percentage of Cry3Bb1 protein as a proportion of total protein in MON 87411 grain is approximately 0.004%, and is calculated as follows:

$$(4.0\ \mu g/g \div 107{,}100\ \mu g/g) \times 100\% \approx 0.004\%\ \text{of total protein}$$

Therefore, the Cry3Bb1 protein represents a very small portion of the total protein in the harvested grain of MON 87411.

B5(e)(ii) The CP4 EPSPS protein as a proportion of total protein

The CP4 EPSPS protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table 22). Among tested tissues of MON 87411, grain is the most relevant to the assessment of food allergenicity since grain is a food and feed source. The mean level of CP4 EPSPS protein in grain of MON 87411 is 1.9 µg/g dw. The mean percent dry weight of total protein in grain of MON 87411 is 10.71% (or 107,100 µg/g). The percentage of CP4 EPSPS protein as a proportion of the total protein in MON 87411 grain is approximately 0.002% and is calculated as follows:

$(1.9\ \mu g/g \div 107{,}100\ \mu g/g) \times 100\% \approx 0.002\%$ or 20 ppm of total protein

Therefore, the CP4 EPSPS protein represents a very small portion of the total protein in harvested grain of MON 87411.

B6 Toxicity of Novel Herbicide Metabolites in GM Herbicide-Tolerant Plants

In Australia, both the import MRL set by FSANZ and the MRL set by the APVMA for glyphosate in corn seed is set at 5 ppm. The use pattern and rate of glyphosate on MON 87411 will follow the existing glyphosate-tolerant corn uses outlined on the glyphosate herbicide label and the glyphosate residues in MON 87411 treated with commercial glyphosate rates are below the established pesticide residue tolerances for corn. Monsanto will not pursue any changes in the established tolerances for its use on MON 87411 corn and because glyphosate metabolite residues have been previously assessed in relation to application A1066, additional data on the identity and levels of herbicide and any metabolites are not given in this application.

B7 Compositional Assessment

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius, 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Compositional assessments are performed using the principles and analytes outlined in the OECD consensus document for maize composition (OECD, 2002a).

A recent review of compositional assessments conducted according to OECD guidelines, which encompassed a total of seven biotechnology-derived crop varieties, nine countries and eleven growing seasons concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition. Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan *et al.*, 2010). Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan *et al.*, 2010; Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Zhou *et al.*, 2011).

Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2002b). OECD consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients. These quantitative measurements effectively discern any compositional changes that imply potential nutritional or safety (e.g. anti-nutritional) concerns. Levels of the components in grain and/or other raw agricultural commodities of the biotechnology-derived crop product are compared to: 1) corresponding levels in a conventional comparator, a genetically similar conventional line, grown concurrently under similar field conditions, and 2) natural ranges generated from an evaluation of commercial reference hybrids grown concurrently and from data published in the scientific literature. The comparison to data published in the literature places any potential differences between the assessed crop and its comparator in the context of the well-documented variation in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites.

Grain and forage samples were collected from MON 87411, the conventional control, and a total of 20 different reference hybrids grown in Argentina during a 2011/2012 field production. The reference hybrids were included in the composition analyses to provide data on the natural variability for each component. The field production was conducted at eight sites. The field sites were planted in a randomized complete block design with four blocks per site. MON 87411, conventional control, and reference hybrids were grown under normal agronomic field conditions for their respective geographic regions, in areas that were typical for maize production in Argentina. Production in the U.S. corn belt and Argentina maize-growing regions occurs at relatively similar latitudes with an approximate 6 month offset (Schnepf *et al.*, 2001). The average growing season temperatures and precipitation are comparable (Schnepf *et al.*, 2001) and maize hybrids developed in the U.S. are often used

directly by farmers in Argentina. As such, compositional analyses from maize grown in Argentina are appropriate for a comparative safety assessment. MON 87411 plots were treated with glyphosate (0.95 kg a.i./ha) to generate samples under conditions of the intended use of the product.

The evaluation of MON 87411 followed considerations relevant to the compositional quality of maize as defined by the OECD consensus document (OECD, 2002a). Grain samples were analyzed for levels of nutrients including proximates, carbohydrates by calculation, fiber, amino acids, fatty acids, minerals, and vitamins. The anti-nutrients analyzed in grain included phytic acid and raffinose. Secondary metabolites analyzed in grain included furfural, ferulic acid, and p-coumaric acid. Forage samples were analyzed for levels of proximates, carbohydrates by calculation, fiber, and minerals. In total, 78 different components were assayed (nine in forage and 69 in grain).

Of those 78 components, 14 fatty acids (caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, pentadecenoic, palmitoleic, heptadecanoic, heptadecenoic, gamma linolenic, eicosadienoic, eicosatrienoic, and arachidonic acids), sodium, and furfural had more than 50% of observations below the assay limit of quantitation (LOQ) and were excluded from statistical analysis. Moisture on two components (grain and forage) was measured for conversion of components to dry weight, but was not statistically analyzed. Therefore, 60 components were statistically analyzed.

The statistical comparison of MON 87411 and the conventional control was based on compositional data combined across all field sites (the combined-site analysis). Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). The compositional data from the reference hybrids were combined across all field sites to calculate a 99% tolerance interval for each component to estimate the natural variability of each component in maize.

A statistically significant difference between MON 87411 and the conventional control does not imply biological relevance from a food and feed safety perspective. Therefore, statistically significant differences observed in the combined-site analysis between MON 87411 and the conventional control were evaluated further to determine whether the detected difference indicated a biologically relevant compositional change or supported a conclusion of compositional equivalence, as follows:

Determination of the mean difference between MON 87411 and the conventional control that was used in steps two and three, below. For protein and amino acids only[6], the relative

[6] Since total amino acids measured in a grain analysis are predominantly derived from hydrolysis of protein, changes in protein levels will likely result in corresponding changes in amino acids levels. However, the mean difference for individual amino acid levels will be less than the mean difference for protein because each amino acid constitutes only a part of the protein. For this reason, the relative magnitudes of difference (percent change relative to the control) for amino acids and protein were determined to allow an assessment of the difference in amino acids in relation to a difference in protein. When the relative magnitudes of difference for amino acids were related to the relative magnitude of difference for protein, then steps 2 and 3 are not discussed for amino acids.

magnitude of the difference (percent change relative to the control) between MON 87411 and the conventional control was determined to allow an assessment of the difference in amino acids in relation to a difference in protein;

Assessment of the relative impact of MON 87411 compared to variation within the conventional control germplasm grown across multiple sites (i.e. variation due to environmental influence). This assessment compares the mean difference between MON 87411 and the conventional control to the range of values for the conventional control (maximum value minus the minimum value) derived from the combined-site analysis. When a mean difference is less than ranges seen due to natural environmental variation within even a single, closely related germplasm, the difference is typically not a food or feed safety concern; and

Assessment of the relative impact of MON 87411 compared to natural variation due to multiple sources (*e.g.*, environmental and germplasm influences). This assessment compares the mean difference between MON 87411 and the conventional control to variation in conventional maize as estimated by in-study reference hybrid values and assessing whether the mean value of MON 87411 was within the 99% tolerance interval, the literature values, and/or the ILSI Crop Composition Database values (ILSI-CCDB) (ILSI, 2011).

These evaluations of natural variation within the context of the conventional control and conventional maize references are important as crop composition is known to be influenced by environment and germplasm. Although used as the first step in the comparative assessment process, detection of statistically significant differences between MON 87411 and conventional control mean values does not imply a meaningful contribution by MON 87411 to compositional variability. Only if mean differences between MON 87411 and the conventional control are large relative to natural variation inherent to conventional maize would further assessments be required to establish whether the change in composition would have an impact from a food and feed safety perspective. The steps reviewed in this assessment therefore describe whether the differences between MON 87411 and the conventional control are meaningful from a food/feed perspective or whether they support a conclusion of compositional equivalence.

The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients, and secondary metabolites in maize grain and forage of MON 87411 and the conventional control. Of the 60 components statistically assessed there were no statistically significant differences in 48 components. Only 12 components (protein, histidine, tyrosine, oleic acid, neutral detergent fiber, copper, iron, manganese, zinc, niacin, vitamin B1 in grain and ash in forage) showed a significant difference between MON 87411 and the conventional control in the combined site analysis. For these 12 components, the mean differences in component values between MON 87411 and the conventional control were less than the observed range of the conventional control values (Step 2 above) and the reference hybrid values (Step 3 above) (Table 27, Table 29, Table 30, Table 31 , and Table 33 below). The MON 87411 mean component values of the 12 components that were statistically different were within the 99% tolerance intervals, the values observed in the literature, and/or the ILSI-CCDB values (Step 3 above). These

results support the overall conclusion that MON 87411 was not a major contributor to variation in component levels in maize grain and forage and confirmed the equivalence of MON 87411 to the conventional control in levels of all measured components. A detailed description of the assessment of statistically significant differences observed between MON 87411 and the conventional control is provided in the following section. These data confirmed that the components with observed significant differences were not compositionally meaningful from a food and feed safety perspective.

Please also refer to Klusmeyer *et al.*, 2013 (MSL0024658).

B7(a) Levels of key nutrients, toxicants and anti-nutrients

Grain samples were analyzed for levels of nutrients including ash, protein, total fat, carbohydrates by calculation, fiber (three components), amino acids (18 components), fatty acids (22 components), minerals (nine components), and vitamins (seven components). Moisture was measured for conversion of components from fresh to dry weight, but was not statistically analyzed.

B7(a)(i) Proteins and amino acids

Maize grain is typically composed of approximately 10% protein and the levels of protein and associated amino acids can vary depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Zhou *et al.*, 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

A statistically significant difference ($p<0.05$) between MON 87411 and the conventional control was observed for protein (Table 27). The mean protein value was 10.71% dw for MON 87411 and 10.28% dw for the conventional control, a mean difference of 0.43% dw. This difference was evaluated in the context of the range of the conventional control values, 4.54% dw, calculated from the minimum (8.06% dw) and maximum (12.60% dw) protein values. The mean difference in protein values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in protein values between MON 87411 and the conventional control was also less than the variation seen in the reference hybrid values (ranged 7.21 to 14.59% dw, a magnitude of 7.38% dw), and the MON 87411 mean value for protein was within the 99% tolerance interval, the values observed in the published literature and/or the ILSI-CCDB values (Table 34).

Since total amino acids measured in a seed analysis are predominantly derived from hydrolysis of protein, differences in amino acid levels between MON 87411 and the conventional control were assessed relative to the difference in protein levels. The relative magnitude of the difference in mean protein values for MON 87411 and the conventional control, when expressed as a percent of the conventional control, was 4.19% (Table 27). Correspondingly, relative magnitudes of difference for the 18 amino acids measured were ≤4.77%. These differences were significant for two of the amino acids (histidine, tyrosine) (Table 27), and reflected small relative magnitudes of differences between MON 87411 and

the conventional control, as would be expected based on the small relative magnitude of difference in protein.

The data demonstrated that MON 87411 was not a major contributor to variation in protein and amino acid levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance intervals, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant differences ($p<0.05$) in mean values of protein and two amino acids were not compositionally meaningful from a food and feed safety perspective.

B7(a)(ii) Total fat and fatty acids

Maize grain is typically composed of approximately 4% fat and the levels of total fat and fatty acid levels can vary depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Zhou *et al.*, 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant difference was observed for total fat (Table 28). There were also no statistically significant differences for seven of the eight fatty acids (FA) assessed. One statistically significant difference ($p<0.05$) was observed for oleic acid (Table 28). The mean oleic acid value was 21.89% total FA for MON 87411 and 21.70% total FA for the conventional control, a difference of 0.19% total FA. This observed statistically significant difference was evaluated in the context of the range of the conventional control values, 2.67% total FA, calculated from the minimum (20.81% total FA) and maximum (23.49% total FA) oleic acid values. The mean difference in oleic acid values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in oleic acid values between MON 87411 and the conventional control was also less than the variation seen in the reference hybrid values (ranged from 20.52 to 42.88% total FA, a magnitude of 22.36% total FA), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

The data demonstrated that MON 87411 was not a major contributor to variation in total fat and fatty acid levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant difference in the mean value of oleic acid was not compositionally meaningful from a food and feed safety perspective.

B7(a)(iii) Carbohydrates by calculation and fiber

In addition to protein and fat, major biomass components assessed in maize grain included carbohydrates by calculation and fiber [acid detergent fiber (ADF), neutral detergent fiber (NDF), and total dietary fiber (TDF)]. Maize grain is typically composed of approximately 85% carbohydrates by calculation, which includes fiber (ADF, NDF, and TDF), and the

levels of these components can vary widely depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Zhou *et al.*, 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant differences were observed for carbohydrates by calculation, ADF, or TDF (Table 29). A statistically significant difference ($p<0.05$) was observed for NDF. The mean NDF value was 8.26% dw for MON 87411 and 8.74% dw for the conventional control, a mean difference of -0.48% dw. This difference was evaluated in the context of the range of the conventional control values, 2.94% dw, calculated from the minimum (7.36% dw) and maximum (10.30% dw) NDF values. The mean difference in NDF values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in NDF values was also less than the variation seen in the reference hybrid values (ranged from 7.41 to 14.80% dw, a magnitude of 7.39% dw), and the MON 87411 mean value for NDF was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

The data demonstrated that MON 87411 was not a major contributor to variation in carbohydrates by calculation and fiber levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant difference in the mean value of NDF was not compositionally meaningful from a food and feed safety perspective.

B7(a)(iv) Ash and minerals

Ash and minerals were also assessed in maize grain. Mineral components (calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc), constituents of ash, are also discussed in this section. The levels of these components can vary widely depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Zhou *et al.*, 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

Ash levels were not statistically significantly different between MON 87411 and the conventional control (Table 30). There were also no statistically significant differences observed for four of the eight minerals assessed, including calcium, magnesium, phosphorus, or potassium. Statistically significant differences ($p<0.05$) were observed between MON 87411 and the conventional control for copper, iron, manganese, and zinc (Table 30).

For copper, the mean value was 1.33 mg/kg dw for MON 87411 and 1.41 mg/kg dw for the conventional control, a difference of -0.077 mg/kg dw. This difference was evaluated in the context of the range of the conventional control values, 0.65 mg/kg dw, calculated from the minimum (1.13 mg/kg dw) and maximum (1.78 mg/kg dw) copper values. The mean difference in copper values between MON 87411 and the conventional control was less than

the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in copper values was also less than the variation seen in the reference hybrid values (ranged 1.10 to 3.23 mg/kg dw, a magnitude of 2.13 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

For iron, the mean value was 16.84 mg/kg dw for MON 87411 and 16.33 mg/kg dw for the conventional control, a difference of 0.51 mg/kg dw. The mean difference in iron values between MON 87411 and the conventional control was less than the range of the conventional control values (4.67 mg/kg dw; 13.72 to 18.39 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in iron values was also less than the variation seen in the reference hybrid values (ranged 13.80 to 24.48 mg/kg dw, a magnitude of 10.68 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

For manganese, the mean value was 6.16 mg/kg dw for MON 87411 and 5.99 mg/kg dw for the conventional control, a difference of 0.17 mg/kg dw. The mean difference in manganese values between MON 87411 and the conventional control was less than the range of the conventional control values (2.68 mg/kg dw; 4.63 to 7.32 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in manganese values was also less than the variation seen in the reference hybrid values (ranged 4.93 to 10.42 mg/kg dw, a magnitude of 5.49 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

For zinc, the mean value was 21.44 mg/kg dw for MON 87411 and 20.93 mg/kg dw for the conventional control, a difference of 0.50 mg/kg dw. The mean difference in zinc values between MON 87411 and the conventional control was less than the range of the conventional control values (6.45 mg/kg dw; 17.76 to 24.21 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in zinc values was also less than the variation seen in the reference hybrid values (ranged 16.40 to 33.92 mg/kg dw, a magnitude of 17.52 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

The data demonstrated that MON 87411 was not a major contributor to variation in ash and mineral levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant differences in mean values of these several minerals were not compositionally meaningful from a food and feed safety perspective.

B7(a)(v) Vitamins

Maize grain contains both water-soluble vitamins (folic acid, niacin, B1, B2, and B6) and fat-soluble vitamins [vitamins A (β-carotene) and E]. The levels of these components can vary widely depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011; Safawo *et al.*, 2010). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant differences were observed for folic acid, vitamin A, vitamin B2, vitamin B6, and vitamin E (Table 31). Statistically significant differences ($p<0.05$) were observed between MON 87411 and the conventional control for niacin and vitamin B1.

For niacin, the mean value was 17.33 mg/kg dw for MON 87411 and 18.78 mg/kg dw for the conventional control, a difference of -1.45 mg/kg dw. This difference was evaluated in the context of the range of the conventional control values, 20.69 mg/kg dw, calculated from the minimum (13.73 mg/kg dw) and maximum (34.41 mg/kg dw) values. The mean difference in niacin values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in niacin values was also less than the variation seen in the reference hybrid values (ranged 14.90 to 38.07 mg/kg dw, a magnitude of 23.17 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

For vitamin B1, the mean value was 3.44 mg/kg dw for MON 87411 and 3.56 mg/kg dw for the conventional control, a difference of -0.12 mg/kg dw. The mean difference in vitamin B1 values between MON 87411 and the conventional control was less than the range of the conventional control values (0.85 mg/kg dw; 3.10 to 3.94 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in vitamin B1 values was also less than the variation seen in the reference hybrid values (ranged 2.79 to 4.96 mg/kg dw, a magnitude of 2.17 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

The data demonstrated that MON 87411 was not a major contributor to variation in vitamin levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant differences in mean values of these vitamins were not compositionally meaningful from a food and feed safety perspective.

B7(a)(vi) Anti-nutrient levels in maize grain

Anti-nutrients assessed included phytic acid and raffinose. Phytic acid, the major storage form of phosphorus in maize grain, is considered an anti-nutrient due to its mineral-chelating properties and the sequestration of phosphorus in phytic acid, reducing phosphorus bioavailabity. Raffinose is a low molecular weight non-digestible carbohydrate that is

considered to be an anti-nutrient due to the enteric gas production and resulting flatulence caused by its consumption (Liener, 2000). The levels of these components can vary widely depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant differences were observed for phytic acid and raffinose (Table 32). The data demonstrated that MON 87411 was not a major contributor to variation in phytic acid and raffinose levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components.

B7(a)(vii) Secondary metabolites levels in maize grain

Secondary metabolites measured in MON 87411 grain included furfural, ferulic acid, and p-coumaric acid according to the OECD consensus document (OECD, 2002a). Furfural was not detected in the grain of MON 87411, the conventional control, or reference hybrids. Ferulic acid and p-coumaric acid are derived from phenylalanine and tyrosine (Buchanan *et al.*, 2000) and serve as precursors for a large group of phenylpropanoid compounds and fiber. The levels of these secondary metabolites can vary widely depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition. No statistically significant differences were observed for ferulic acid or p-coumaric acid (Table 32). The data demonstrated that MON 87411 was not a major contributor to variation in ferulic acid and p-coumaric acid levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components.

B7(a)(viii) Nutrient levels in maize forage

Forage samples were assessed for levels of ash, protein, total fat, carbohydrates by calculation, fiber (ADF and NDF), and minerals (calcium and phosphorus). The levels of these components can vary widely depending on local growing conditions (Harrigan *et al.*, 2009; Ridley *et al.*, 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

With the exception of ash, there were no statistically significant differences in levels of proximates, fiber, or minerals (Table 33). The mean ash value was 5.57% dw for MON 87411 and 5.95% dw for the conventional control, a difference of -0.39% dw. This difference was evaluated in the context of the range of the conventional control values, 5.04% dw, calculated from the minimum (4.51% dw) and maximum (9.55% dw) values. The mean difference in ash values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference was also less than the variation seen in the reference values (ranged 3.53 to 8.33% dw, a magnitude of 4.80% dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table 34).

The data demonstrated that MON 87411 was not a major contributor to variation in ash levels in maize forage and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean value of this component was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the observed significant difference in mean value of ash was not compositionally meaningful from a food and feed safety perspective.

Table 27 Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

					Difference (Test minus Control)		
Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Mean (S.E.)	p-Value	% Relative[6]
Protein	10.71 (0.49) 8.03 - 13.10	10.28 (0.49) 8.06 - 12.60	(7.21 - 14.59) 5.66, 15.13	4.54	0.43 (0.17)	0.023	4.19
Alanine	0.84 (0.044) 0.62 - 1.03	0.81 (0.044) 0.61 - 1.03	(0.51 - 1.20) 0.37, 1.24	0.42	0.027 (0.019)	0.166	3.34
Arginine	0.48 (0.013) 0.41 - 0.54	0.47 (0.013) 0.40 - 0.54	(0.40 - 0.66) 0.35, 0.66	0.15	0.0088 (0.0055)	0.133	1.86
Aspartic Acid	0.68 (0.028) 0.54 - 0.80	0.66 (0.028) 0.52 - 0.80	(0.49 - 0.91) 0.42, 0.91	0.28	0.022 (0.011)	0.068	3.41
Cystine/Cysteine	0.21 (0.0061) 0.17 - 0.25	0.21 (0.0061) 0.17 - 0.24	(0.16 - 0.30) 0.12, 0.32	0.07	0.0010 (0.0034)	0.768	0.50
Glutamic Acid	2.03 (0.11) 1.44 - 2.51	1.95 (0.11) 1.43 - 2.49	(1.23 - 2.87) 0.87, 3.02	1.06	0.078 (0.045)	0.108	3.98

Table 27 (continued) Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value	% Relative[6]
Glycine	0.38 (0.0099) 0.34 - 0.43	0.38 (0.0099) 0.32 - 0.44	(0.31 - 0.51) 0.27, 0.52	0.12	0.0024 (0.0044)	0.591	0.64
Histidine	0.28 (0.010) 0.23 - 0.32	0.27 (0.010) 0.21 - 0.34	(0.20 - 0.46) 0.13, 0.45	0.12	0.0096 (0.0041)	0.033	3.54
Isoleucine	0.40 (0.020) 0.29 - 0.49	0.38 (0.020) 0.27 - 0.48	(0.25 - 0.54) 0.19, 0.56	0.21	0.018 (0.0084)	0.050	4.77
Leucine	1.41 (0.086) 0.96 - 1.79	1.35 (0.086) 0.95 - 1.76	(0.79 - 2.01) 0.51, 2.14	0.81	0.061 (0.034)	0.100	4.50
Lysine	0.28 (0.0057) 0.24 - 0.32	0.27 (0.0057) 0.22 - 0.31	(0.24 - 0.34) 0.22, 0.35	0.09	0.0057 (0.0042)	0.197	2.12
Methionine	0.21 (0.0074) 0.18 - 0.25	0.21 (0.0074) 0.17 - 0.25	(0.15 - 0.27) 0.13, 0.28	0.08	-0.00024 (0.0040)	0.952	-0.11

Table 27 (continued) Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

					Difference (Test minus Control)		
Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Mean (S.E.)	p-Value	% Relative[6]
Phenylalanine	0.56 (0.032) 0.41 - 0.70	0.54 (0.032) 0.40 - 0.70	(0.34 - 0.78) 0.24, 0.83	0.31	0.023 (0.014)	0.130	4.20
Proline	0.97 (0.045) 0.74 - 1.18	0.95 (0.045) 0.74 - 1.15	(0.64 - 1.39) 0.44, 1.47	0.41	0.019 (0.018)	0.321	1.98
Serine	0.48 (0.023) 0.35 - 0.60	0.47 (0.023) 0.33 - 0.59	(0.33 - 0.67) 0.25, 0.69	0.26	0.017 (0.0093)	0.095	3.53
Threonine	0.37 (0.015) 0.30 - 0.44	0.36 (0.015) 0.29 - 0.44	(0.27 - 0.50) 0.22, 0.51	0.14	0.010 (0.0062)	0.131	2.74
Tryptophan	0.071 (0.0016) 0.064 - 0.080	0.071 (0.0016) 0.059 - 0.082	(0.053 - 0.086) 0.053, 0.091	0.02	0.00018 (0.00070)	0.805	0.25
Tyrosine	0.42 (0.020) 0.31 - 0.52	0.40 (0.020) 0.30 - 0.52	(0.27 - 0.57) 0.22, 0.58	0.22	0.018 (0.0082)	0.046	4.42

Table 27 (continued) Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

					Difference (Test minus Control)		
Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Mean (S.E.)	p-Value	% Relative[6]
Valine	0.49 (0.021) 0.39 - 0.58	0.48 (0.021) 0.37 - 0.59	(0.34 - 0.70) 0.27, 0.70	0.22	0.015 (0.010)	0.167	3.04

[1]dw = dry weight.

[2]Mean (S.E.) = least-square mean (standard error).

[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.

[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of reference hybrids. Negative limits were set to zero.

[5]Maximum value minus minimum value for the conventional control maize hybrid.

[6]The relative magnitude of the difference in mean values between MON 87411 and the conventional control, expressed as a percent of the conventional control.

Table 28 Summary of Maize Grain Fat and Fatty Acids for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Total Fat (% dw)[1]	3.79 (0.047) 3.42 - 4.15	3.83 (0.047) 3.43 - 4.10	(2.65 - 5.60) 1.55, 6.69	0.68	-0.036 (0.063)	0.573
16:0 Palmitic[6]	13.61 (0.045) 13.31 - 13.97	13.62 (0.045) 13.32 - 14.05	(9.98 - 12.71) 8.50, 14.14	0.73	-0.0063 (0.032)	0.842
18:0 Stearic	1.68 (0.032) 1.57 - 1.88	1.70 (0.032) 1.53 - 1.96	(1.56 - 2.58) 0.93, 2.98	0.43	-0.021 (0.018)	0.249
18:1 Oleic	21.89 (0.15) 20.86 - 22.96	21.70 (0.15) 20.81 - 23.49	(20.52 - 42.88) 7.74, 50.71	2.67	0.19 (0.091)	0.040
18:2 Linoleic	60.90 (0.22) 59.38 - 61.96	61.06 (0.22) 59.08 - 62.38	(42.82 - 64.10) 33.63, 77.43	3.30	-0.17 (0.099)	0.095
18:3 Linolenic	1.09 (0.0093) 1.04 - 1.17	1.09 (0.0093) 1.02 - 1.16	(0.85 - 1.41) 0.57, 1.65	0.14	0.0035 (0.0059)	0.552

Table 28 (continued) Summary of Maize Grain Fat and Fatty Acids for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
20:0 Arachidic	0.41 (0.0068) 0.39 - 0.44	0.42 (0.0068) 0.37 - 0.47	(0.33 - 0.63) 0.21, 0.70	0.09	-0.0024 (0.0042)	0.571
20:1 Eicosenoic	0.26 (0.0018) 0.24 - 0.28	0.26 (0.0018) 0.24 - 0.27	(0.19 - 0.34) 0.12, 0.38	0.03	0.0038 (0.0022)	0.101
22:0 Behenic	0.16 (0.0016) 0.14 - 0.17	0.16 (0.0016) 0.15 - 0.18	(0.055 - 0.25) 0.0065, 0.31	0.03	-0.0016 (0.0020)	0.434

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference maize hybrids. Negative limits set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.
[6]Fatty acid means and ranges are expressed as a % of total fatty acid. Prefix numbers refer to number of carbon atoms and number of carbon-carbon double bonds in the fatty acid molecule; 16:0 means sixteen carbon atoms and zero double bonds.
Numbers are not included in text discussion for reasons of clarity. The following fatty acids with more than 50% of observations below the assay LOQ were excluded from statistical analysis: caprylic acid, capric acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid, pentadecenoic acid, palmitoleic acid, heptadecanoic acid, heptadecenoic acid, gamma linolenic acid, eicosadienoic acid, eicosatrienoic acid, and arachidonic acid.

Table 29 Summary of Maize Grain Carbohydrates by Calculation and Fiber for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Carbohydrates by	84.13 (0.51)	84.53 (0.51)	(78.76 - 87.56)	4.77	-0.40 (0.20)	0.068
Calculation	81.84 - 86.85	82.18 - 86.95	77.72, 90.40			
Acid Detergent Fiber	3.06 (0.083)	3.26 (0.083)	(1.89 - 5.16)	1.26	-0.20 (0.10)	0.074
	2.50 - 3.62	2.73 - 4.00	2.18, 4.98			
Neutral Detergent Fiber	8.26 (0.17)	8.74 (0.17)	(7.41 - 14.80)	2.94	-0.48 (0.20)	0.018
	7.12 - 12.14	7.36 - 10.30	6.04, 13.44			
Total Dietary Fiber	11.50 (0.20)	11.82 (0.20)	(10.33 - 17.11)	4.60	-0.33 (0.27)	0.247
	10.19 - 12.90	10.43 - 15.03	9.83, 16.84			

[1]dw = dry weight.

[2]Mean (S.E.) = least-square mean (standard error).

[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.

[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference maize hybrids. Negative limits set to zero.

[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table 30 Summary of Maize Grain Ash and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Ash (% dw)[1]	1.36 (0.026) 1.13 - 1.51	1.35 (0.026) 1.05 - 1.60	(1.07 - 1.75) 1.07, 1.80	0.55	0.0098 (0.030)	0.746
Calcium (% dw)	0.0031 (0.00007) 0.0024 - 0.0037	0.0030 (0.00007) 0.0026 - 0.0033	(0.0026 - 0.0056) 0.0019, 0.0062	0.00	0.00009 (0.00005)	0.063
Copper (mg/kg dw)	1.33 (0.056) 1.09 - 1.88	1.41 (0.056) 1.13 - 1.78	(1.10 - 3.23) 0.28, 3.75	0.65	-0.077 (0.030)	0.021
Iron (mg/kg dw)	16.84 (0.41) 14.59 - 19.70	16.33 (0.41) 13.72 - 18.39	(13.80 - 24.48) 10.71, 28.62	4.67	0.51 (0.18)	0.013
Magnesium (% dw)	0.11 (0.0031) 0.094 - 0.13	0.12 (0.0031) 0.094 - 0.13	(0.097 - 0.15) 0.086, 0.16	0.03	-0.00050 (0.0011)	0.657
Manganese (mg/kg dw)	6.16 (0.26) 4.64 - 7.13	5.99 (0.26) 4.63 - 7.32	(4.93 - 10.42) 2.28, 12.14	2.68	0.17 (0.070)	0.033

Table 30 (continued) Summary of Maize Grain Ash and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Phosphorus (% dw)	0.31 (0.0071)	0.31 (0.0071)	(0.24 - 0.39)	0.11	-0.0018 (0.0033)	0.591
	0.25 - 0.33	0.23 - 0.34	0.22, 0.43			
Potassium (% dw)	0.34 (0.0061)	0.35 (0.0061)	(0.30 - 0.43)	0.09	-0.0055 (0.0034)	0.127
	0.32 - 0.39	0.31 - 0.40	0.28, 0.46			
Zinc (mg/kg dw)	21.44 (0.72)	20.93 (0.72)	(16.40 - 33.92)	6.45	0.50 (0.22)	0.038
	18.54 - 26.54	17.76 - 24.21	11.63, 36.32			

[1]dw = dry weight.

[2]Mean (S.E.) = least-square mean (standard error).

[3]Range is the minimum and maximum raw values for the conventional reference hybrids.

[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.

[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table 31 Summary of Maize Grain Vitamins for MON 87411, Conventional Control, and Reference Hybrids

Component (mg/kg dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Folic Acid	0.28 (0.0071) 0.23 - 0.39	0.28 (0.0071) 0.21 - 0.37	(0.19 - 0.52) 0.084, 0.56	0.17	0.0041 (0.0074)	0.580
Niacin	17.33 (0.75) 14.86 - 20.70	18.78 (0.75) 13.73 - 34.41	(14.90 - 38.07) 4.69, 42.03	20.69	-1.45 (0.61)	0.021
Vitamin A	1.29 (0.058) 0.75 - 1.57	1.38 (0.058) 0.65 - 3.59	(0.32 - 4.76) 0, 4.91	2.94	-0.095 (0.064)	0.145
Vitamin B1	3.44 (0.065) 3.04 - 3.85	3.56 (0.065) 3.10 - 3.94	(2.79 - 4.96) 1.86, 5.07	0.85	-0.12 (0.047)	0.021
Vitamin B2	1.53 (0.048) 1.22 - 2.03	1.64 (0.048) 1.28 - 2.30	(1.15 - 2.54) 0.94, 2.37	1.01	-0.11 (0.052)	0.058
Vitamin B6	6.16 (0.11) 5.15 - 7.44	6.10 (0.11) 5.07 - 8.56	(5.09 - 12.13) 3.84, 10.03	3.49	0.060 (0.13)	0.648

Table 31 (continued) Summary of Maize Grain Vitamins for MON 87411, Conventional Control, and Reference Hybrids

Component (mg/kg dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Vitamin E	10.53 (0.24)	10.28 (0.24)	(6.37 - 31.91)	3.78	0.25 (0.29)	0.400
	6.99 - 15.33	8.94 - 12.72	0, 30.69			

[1]dw = dry weight.

[2]Mean (S.E.) = least-square mean (standard error).

[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.

[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.

[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table 32 Summary of Maize Grain Anti-nutrients and Secondary Metabolites for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Anti-nutrient (% dw[1])						
Phytic Acid	0.99 (0.029) 0.75 - 1.27	0.98 (0.029) 0.67 - 1.19	(0.60 - 1.25) 0.56, 1.41	0.52	0.012 (0.022)	0.584
Raffinose	0.25 (0.0081) 0.14 - 0.31	0.24 (0.0081) 0.10 - 0.29	(0.093 - 0.40) 0, 0.45	0.19	0.0066 (0.0058)	0.256
Secondary Metabolite (µg/g dw)						
Ferulic Acid	1846.74 (28.24) 1528.09 - 2031.96	1896.61 (28.24) 1700.68 - 2093.02	(1337.21 - 3286.55) 749.39, 3421.84	392.34	-49.87 (29.30)	0.110
p-Coumaric Acid	148.56 (2.91) 132.88 - 197.75	148.27 (2.91) 131.03 - 164.37	(62.24 - 387.51) 0, 461.05	33.33	0.28 (3.31)	0.932

[1]dw = dry weight.

[2]Mean (S.E.) = least-square mean (standard error).

[3]Range is the minimum and maximum raw values for the conventional reference hybrids.

[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.

[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table 33 Summary of Maize Forage Proximates, Fiber and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Ash	5.57 (0.22)	5.95 (0.22)	(3.53 - 8.33)	5.04	-0.39 (0.16)	0.018
	3.95 - 7.01	4.51 - 9.55	3.38, 7.59			
Carbohydrates by	84.96 (0.56)	84.57 (0.56)	(79.74 - 90.37)	7.09	0.38 (0.25)	0.128
Calculation	81.65 - 88.55	80.91 - 88.00	80.32, 89.79			
Protein	7.58 (0.46)	7.42 (0.46)	(4.19 - 11.38)	5.03	0.17 (0.16)	0.321
	5.27 - 10.25	4.63 - 9.66	3.12, 11.55			
Total Fat	1.91 (0.17)	2.07 (0.17)	(0.66 - 3.60)	2.40	-0.16 (0.15)	0.296
	0.49 - 2.93	0.99 - 3.39	0.67, 3.58			
Acid Detergent Fiber	25.45 (0.94)	25.07 (0.94)	(18.64 - 37.68)	12.65	0.39 (0.66)	0.558
	16.58 - 34.25	17.89 - 30.55	14.75, 38.41			
Neutral Detergent Fiber	44.68 (1.49)	42.84 (1.49)	(34.97 - 67.39)	19.17	1.84 (1.19)	0.145
	28.32 - 55.35	32.16 - 51.33	28.74, 62.39			

Table 33 (continued) Summary of Maize Forage Proximates, Fiber and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Calcium	0.17 (0.016) 0.093 - 0.26	0.18 (0.016) 0.084 - 0.42	(0.074 - 0.37) 0.015, 0.32	0.34	-0.013 (0.0078)	0.112
Phosphorus	0.19 (0.011) 0.095 - 0.24	0.19 (0.011) 0.13 - 0.25	(0.11 - 0.26) 0.082, 0.26	0.13	-0.00002 (0.0058)	0.997

[1]dw = dry weight.

[2]Mean (S.E.) = least-square mean (standard error).

[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.

[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.

[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table 34 Literature and ILSI Database Ranges for Components in Maize Forage and Grain

Grain Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Grain Nutrients		
Proximates (% dw)		
Ash	1.17 – 2.01[a]; 1.27 – 1.63[b]	0.616 – 6.282
Carbohydrates by calculation	81.31 – 87.06[a]; 82.10 – 85.98[b]	77.4 – 89.5
Fat, total	2.95 – 4.40[a]; 3.18 – 4.23[b]	1.742 – 5.900
Protein	8.27 – 13.33[a]; 9.17 – 12.19[b]	6.15 – 17.26
Fiber (% dw)		
Acid detergent fiber	1.82 – 4.48[a]; 1.83 – 3.39[b]	1.82 – 11.34
Neutral detergent fiber	6.51 –12.28[a]; 6.08 – 10.36[b]	5.59 – 22.64
Total dietary fiber	10.65 – 16.26[a]; 10.57 – 14.56[b]	9.01 – 35.31
Amino Acids (% dw)		
Alanine	0.60 – 1.04[a]; 0.68 – 0.96[b]	0.44 - 1.39
Arginine	0.34 – 0.52[a]; 0.34 – 0.50[b]	0.12 - 0.64
Aspartic acid	0.52 – 0.78[a]; 0.59 – 0.76[b]	0.33 – 1.21
Cystine	0.19 – 0.26[a]; 0.20 – 0.26[b]	0.13 – 0.51
Glutamic acid	1.54 – 2.67[a]; 1.71 – 2.44[b]	0.97 – 3.54
Glycine	0.33 – 0.43[a]; 0.33 – 0.42[b]	0.18 – 0.54
Histidine	0.25 – 0.37[a]; 0.27 – 0.34[b]	0.14 – 0.43
Isoleucine	0.30 – 0.48[a]; 0.32 – 0.44[b]	0.18 – 0.69
Leucine	1.02 – 1.87[a]; 1.13 – 1.65[b]	0.64 – 2.49
Lysine	0.26 – 0.33[a]; 0.28 – 0.31[b]	0.17 – 0.67
Methionine	0.17 – 0.26[a]; 0.16 – 0.30[b]	0.12 – 0.47
Phenylalanine	0.43 – 0.72[a]; 0.45 – 0.63[b]	0.24 – 0.93
Proline	0.74 – 1.21[a]; 0.78 – 1.11[b]	0.46 – 1.63
Serine	0.39 – 0.67[a]; 0.43 – 0.60[b]	0.24 – 0.77
Threonine	0.29 – 0.45[a]; 0.31 – 0.39[b]	0.22 – 0.67
Tryptophan	0.047 – 0.085[a]; 0.042 – 0.070[b]	0.027 – 0.215
Tyrosine	0.13 – 0.43[a]; 0.12 – 0.41[b]	0.10 – 0.64
Valine	0.42 – 0.62[a]; 0.45 – 0.58[b]	0.27 – 0.86
Fatty Acids (% Total FA)		
16:0 Palmitic	8.80 – 13.33[a]; 9.84 – 12.33[b]	7.94 – 20.71
18:0 Stearic	1.36 – 2.14 [a]; 1.30 – 2.10[b]	1.02 – 3.40
18:1 Oleic	19.50 – 33.71[a]; 19.59 – 29.13[b]	17.4 – 40.2
18:2 Linoleic	49.31 – 64.70[a]; 56.51 – 65.65[b]	36.2 – 66.5
18:3 Linolenic	0.89 – 1.56[a]; 1.03 – 1.38[b]	0.57 – 2.25
20:0 Arachidic	0.30 – 0.49[a]; 0.30 – 0.41[b]	0.279 – 0.965
20:1 Eicosenoic	0.17 – 0.29[a]; 0.17 – 0.27[b]	0.170 – 1.917
22:0 Behenic	0.069 – 0.28[a]; 0.059 – 0.18[b]	0.110 – 0.349
Minerals		
Calcium (% dw)	0.0036 – 0.0068[a]; 0.0035 – 0.0070[b]	0.00127 – 0.02084
Copper (mg/kg dw)	1.14 – 3.43[a]; 1.39 – 2.76[b]	0.73 – 18.50
Iron (mg/kg dw)	14.17 – 23.40[a]; 15.90 – 24.66[b]	10.42 – 49.07
Magnesium (% dw)	0.091 – 0.14[a]; 0.10 – 0.14[b]	0.0594 – 0.194
Manganese (mg/kg dw)	4.83 – 8.34[a]; 4.78 – 9.35[b]	1.69 – 14.30
Phosphorous (% dw)	0.24 – 0.37[a]; 0.27 – 0.38[b]	0.147 – 0.533
Potassium (% dw)	0.29 – 0.39[a]; 0.36 – 0.43[b]	0.181 – 0.603
Zinc (mg/kg dw)	16.78 – 28.17[a]; 18.25 – 30.44[b]	6.5 – 37.2

Table 34 (continued) Literature and ILSI Database Ranges for Components in Maize Forage and Grain

Grain Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Vitamins (mg/kg dw)		
Folic acid	0.19 – 0.35[a]; 0.23 – 0.42[b]	0.147 – 1.464
Vitamin A [β–Carotene]	122 – 4740[c]	0.19 – 46.81
Vitamin B_1 [Thiamine]	2.33 – 4.17[a]; 2.71 – 4.33[b]	1.26 – 40.00
Vitamin B_2 [Riboflavin]	0.94 – 2.42[a]; 1.64 – 2.81[b]	0.50 – 2.36
Vitamin B_3 [Niacin]	15.07 – 32.38[a]; 13.64 – 42.06[b]	10.37 – 46.94
Vitamin B_6 [Pyridoxine]	4.93 – 7.53[a]; 4.97 – 8.27[b]	3.68 – 11.32
Vitamin E [α–Tocopherol]	5.96 – 18.44[a]; 2.84 – 15.53[b]	1.537 – 68.672
Grain Anti–Nutrients (% dw)		
Phytic acid	0.69 – 1.09[a]; 0.60 – 0.94[b]	0.111 – 1.570
Raffinose	0.079 – 0.22[a]; 0.061 – 0.15[b]	0.020 – 0.320
Grain Secondary Metabolites (μg/g dw)		
Ferulic acid	1205.75 – 2873.05[a]; 1011.40 – 2539.86[b]	291.9 – 3885.8
p–Coumaric acid	94.77 – 327.39[a]; 66.48 – 259.68[b]	53.4 – 576.2

Forage Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Forage Nutrients		
Proximates (% dw)		
Ash	2.67 – 8.01[a]; 4.59 – 6.90[b]	1.527 – 9.638
Carbohydrates by calculation	81.88 – 89.26[a]; 84.11 – 87.54[b]	76.4 – 92.1
Fat, total	1.28 – 3.62[a]; 0.20 – 1.76[b]	0.296 – 4.570
Protein	5.80 – 10.24[a]; 5.56 – 9.14[b]	3.14 – 11.57
Fiber (% dw)		
Acid detergent fiber	19.11 – 30.49[a]; 20.73 – 33.39[b]	16.13 – 47.39
Neutral detergent fiber	27.73 – 49.62[a]; 31.81 – 50.61[b]	20.29 – 63.71
Minerals (% dw)		
Calcium	0.12 – 0.33[a]; 0.21 – 0.41[b]	0.07139 – 0.57679
Phosphorous	0.090 – 0.26[a]; 0.13 – 0.21[b]	0.09362 – 0.37041

[1]dw=dry weight; FA = fatty acids.

[2]Literature range references: [a]US and [b]Chile (Harrigan *et al.*, 2009), [c](Safawo *et al.*, 2010).

[3]ILSI range is from ILSI Crop Composition Database, 2011 [Accessed 9 January 2013] (ILSI, 2011).

Compositional assessment of MON 87411 - Conclusion

Compositional analysis was conducted on grain and forage of MON 87411 treated with glyphosate grown at eight sites in a 2011/2012 field production in Argentina that are representative of commercial agricultural regions for maize production. The compositional analysis, based on the OECD consensus document for maize, included measurement of nutrients, anti-nutrients and secondary metabolites in conventional commercial reference hybrids to provide data on the natural variability of each compositional component analyzed.

Of the 60 components statistically assessed for MON 87411, only 12 components (protein, histidine, tyrosine, oleic acid, neutral detergent fiber, copper, iron, manganese, zinc, niacin, and vitamin B1 in grain and ash in forage) showed a significant difference between MON 87411 and the conventional control in the combined-site analysis. For these 12 components, the mean difference in component values between MON 87411 and the conventional control was less than the range of the conventional control values and the reference hybrid values. For all components analyzed, the MON 87411 mean component values were within the tolerance intervals, the values observed in the literature, and/or the ILSI-CCDB values.

These results support the overall conclusion that MON 87411 is not a major contributor to variation in component levels in maize grain and forage and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. These data confirmed that the components with a statistically significant differences ($p<0.05$) between MON 87411 and the conventional control were not compositionally meaningful from a food and feed safety perspective.

B7(b) Levels of other GM-influenced constituents

Not applicable.

B7(c) Levels of naturally-occurring allergenic proteins

Not applicable.

C Nutritional Impact

C1 Data on Nutritional Impact of Compositional Changes

Not Applicable.

C2 Data from an Animal Feeding Study, if Available

The data and information presented in this submission demonstrate that the food and feed derived from MON 87411 are as safe and nutritious as those derived from commercially-available, conventional corn for which there is an established history of safe consumption. Therefore, animal feeding studies do not add value to the safety of MON 87411.

PART 3 STATUTORY DECLARATION – AUSTRALIA

I, Nina McCormick, declare that the information provided in this application fully sets out the matters required and that the same are true to the best of my knowledge and belief, and that no information has been withheld that might prejudice this application.

Signature: _____

Declared before me ……

This ……4th…….. day of ……July…….. 2014.

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