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The Standards Liaison Officer
Australia New Zealand Food Authority
Macquarie House
55 Blackall Street
Barton ACT 2600

Dear Sir

AUSTRALIA NEW ZEALAND FOOD AUTHORITY ACT 1991

Corn Rootworm Protected Corn Event MON863
YieldGard® Rootworm Corn Seed

Please find enclosed an application for Corn Rootworm Protected Corn Event MON863, requesting inclusion of this product in the Food Standard 1.5.2 - Food Derived From Gene Technology. The first volume of the submission contains the application, and the subsequent volumes contain the supporting studies referenced in the application. In line with previous discussions with ANZFA, four copies of the application, and three copies of the supporting studies have been provided.

An additional confidential attachment has been supplied. This volume refers to confidential information that has been deleted from the supporting studies. The confidential information is from the supporting studies numbered 2. (Vol 2), 5. and 6 (Vol 3). We request this attachment volume be kept confidential. Only one copy has been supplied.

Corn from Corn Rootworm Protected Corn Event MON863 are classified as Group D for safety assessment and according to the Assessment Guidelines for Standard 1.5.2. Analysis of the Group D safety assessment leads to a conclusion of "permit use" as outlined in the flow chart of the Guidelines. Attached is a copy of the flow chart to indicate the pathway applicable to Corn Rootworm Protected Corn Event MON863. A checklist relevant to this application is also enclosed. A cheque for $58,800 has been enclosed being $2,800 for the initial assessment fee and $56,000 for the Category 4 – Complex Draft and Final assessment fee.

Yours Sincerely
MONSANTO AUSTRALIA LIMITED

Megan Shaw
Regulatory Product Manager
02 Dec 2002

A484

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APPLICANT: Monsanto Australia Limited

Corn Rootworm Protected Corn Event MON863

SUBMISSION: Application to Food Standards Australia New Zealand for the inclusion of corn containing the Cry3Bb1 gene by Monsanto in Standard 1.5.2 - Food Derived From Gene Technology

VOLUME: 1 of 5

DATE: 02 December 2002

PREPARED BY: Megan Shaw
Regulatory Affairs

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ANZFA Food Standard 1.5.2 Application

Corn Rootworm Protected Corn Event MON 863

Submitted By

Monsanto Australia Ltd.
P.O. Box 6051
St. Kilda Road Central
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Date

7 November 2002

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1.0 KEY TO ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid herbicide
35S	Cauliflower mosaic virus (CaMV) promoter
A1	Monsanto proprietary inbred line of *Zea mays*
A634	Publicly available inbred line of *Zea mays*
ADF	Acid detergent fiber
ANZFA	Australia New Zealand Food Authority
AS1	Activating sequence-1
B14	Publicly available inbred line of *Zea mays*
BC	Back cross to a recurrent nontransgenic parent
BC_1F_1	First filial generation derived from back crossing to a recurrent nontransgenic parent
BC_2F_1	First filial generation derived from a second back crossing to a recurrent nontransgenic parent
BC_2F_2	Second filial generation derived from a second back crossing to a recurrent nontransgenic parent
ble	Gene that encodes bleomycin binding protein from *E. coli* transposon Tn5
BLE	Bleomycin binding protein
bp	Base pairs
B.t.	*Bacillus thuringiensis*
B.t.k.	*Bacillus thuringiensis* subspecies *kumamotoensis*
CaMV	Cauliflower mosaic virus
CFU	Colony forming units
C.I.	Confidence interval
CRW	Corn rootworm, *Diabrotica* sp.
Cry	Crystal protein, a diverse group of insecticidal proteins produced by *B.t.*
Cry1	Class of Lepidopteran-specific *B.t.* Cry proteins that share >45% amino acid sequence homology
Cry1A	Class of Lepidopteran-specific *B.t.* Cry1 proteins that share >75% amino acid sequence homology
Cry1Aa	Class of Lepidopteran-specific *B.t.* Cry1A proteins that share >95% amino acid sequence homology
Cry1Ab	Class of Lepidopteran-specific *B.t.* Cry1A proteins that share >95% amino acid sequence homology
Cry1Ac	Class of Lepidopteran-specific *B.t.* Cry1A proteins that share >95% amino acid sequence homology
Cry3	Class of Coleopteran-specific *B.t.* Cry proteins that share >45% amino acid sequence homology
Cry3Aa	Class of Coleopteran-specific *B.t.* Cry3 proteins that share >95% amino acid sequence homology
Cry3Aa4	Commercially available Cry3A protein that is active against the Colorado potato beetle
CryIIIB2	Outdated designation for the Cry3Bb proteins
Cry3Bb1	Natural isolate, and holotype, of the Cry3Bb class of *B.t.* Cry proteins. This protein is present in the microbial spray product, *Raven Oil Flowable Bioinsecticide*

cry3Bb1	DNA sequence that encodes the protein, Cry3Bb1 (GenBank Accession No. M89794)
DDE	Daily dietary exposure
df	Degrees of freedom
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
Eco RV	Restriction endonuclease that cuts DNA at specific locations
ELISA	Enzyme-linked immunosorbent assay
EG4691	A native strain of *B.t.* that produces Cry3Bb1 protein
EG11098	A recombinant strain of *B.t.* that produces a variant of the wild type Cry3Bb1 protein
EG11231	A recombinant strain of *B.t.* that produces a variant of the wild type Cry3Bb1 protein
EPA	Environmental Protection Agency (U.S.)
F_1	First filial generation
F_2	Second filial generation
FDA	Food and Drug Administration (U.S.)
fw	Fresh weight
Hind III	Restriction endonuclease that cuts DNA at specific locations
kb	Kilobase pairs
kDa	Kilodaltons
Mlu I	Restriction endonuclease that cuts DNA at specific locations
MN	Monmouth, Illinois site used for production of MON 863 tissue samples
Mo17	Publicly available inbred line of *Zea mays*
MOS	Margin of safety
MON 846	Nontransgenic parental hybrid corn line (A634 x A1)
MON 863	Transgenic corn event that expresses a variant of the insecticidal protein, Cry3Bb1. The variant produced in corn event MON 863 differs from wild type Cry3Bb1 protein by 7 amino acids.
mRNA	Messenger RNA
MW	Molecular weight
NASS	National Agricultural Statistics Service (U.S.)
NCGA	National Corn Grower's Association (U.S.)
Nco I	Restriction endonuclease that cuts DNA at specific locations
Nde I	Restriction endonuclease that cuts DNA at specific locations
NDF	Neutral detergent fiber
NOEL	No observable effect level
NOS 3'	Nopaline synthase 3' transcription termination sequence
nptII	DNA sequence that encodes for the enzyme neomycin phosphotransferase type II
NPTII	Neomycin phosphotransferase type II enzyme
ORF	Open reading frame
ori-pUC	Origin of replication for pUC plasmid
p	Probability
PCR	Polymerase chain reaction
ppm	Parts per million

PV-ZMIR13	Plasmid containing the cry3Bb1 and *nptII* gene cassettes
ract1 intron	Intron from the rice actin gene
RD	Richland, Iowa site used for production of MON 863 tissue samples
RNA	Ribonucleic acid
R_0	Designation for originally transformed plant
SD	Standard deviation
sp	Species
subsp.	Subspecies
tahsp17 3'	3' nontranslated sequence of wheat heat shock protein 17.3
T-DNA	Transfer DNA
T.I.	Tolerance interval
Tn5	Transposon 5
U.S.	United States of America
USDA	United States Department of Agriculture
US$	U.S. dollars
VH	Van Horne, Iowa site used for production of MON 863 tissue samples
WTO	World Trade Organization
wt CAB	5' untranslated leader sequence of wheat chlorophyll a/b-binding protein
YK	York, Nebraska site used for production of MON 863 tissue samples
ZMIR13L	Linear fragment of the plasmid PV-ZMIR13 used in transformation to produce corn event MON 863

Standard abbreviations (*e.g.*, units of measure) are used according to the format described in 'Instructions to Authors' in the Journal of Biological Chemistry.

2.0 GENERAL INFORMATION

2.1 APPLICANT

Company Name: Monsanto Australia Limited

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Nature of Your Business: Technology provider to the Agricultural and Food Industries.

2.2 NATURE OF APPLICATION

This application is on behalf of a single firm. This application is not made on behalf of any other party.

The purpose of this submission is to allow the Australia New Zealand Food Authority (ANZFA) to conduct a safety assessment on Corn Rootworm Protected Corn Event MON 863 (hereafter referred to as MON 863). This submission seeks the addition of MON 863 corn and products containing MON 863 corn in the table to Standard 1.5.2 - Food Produced Using Gene Technology.

Column 1 **Food derived from gene technology**	**Column 2** **Special requirements**
Corn Rootworm Protected Corn Event MON 863	None
Products derived from Corn Rootworm Protected Corn Event MON 863	None

3.0 SAFETY ASSESSMENT DATA

3.1 BACKGROUND DETAILS

3.1.1 Description of the New Genetically Modified (GM) Organism

Monsanto Company has developed, through the use of recombinant DNA techniques, corn plants that are protected from damage due to corn rootworm (CRW) feeding. The tissues of these plants produce a modified *Bacillus thuringiensis (*subspecies *kumamotoensis)* Cry3Bb1 protein that is selectively toxic to CRW species (*Diabrotica* spp.). A synthetic variant of the *cry3Bb1* gene was constructed by Monsanto and incorporated into transformation vector, ZMIR13L. Transformation of plants with this vector resulted in the creation of corn event MON 863. Corn varieties containing transformation event MON 863 are afforded a level of protection from CRW feeding damage that is comparable or superior to that offered by currently available conventional insecticides.

Event MON 863 was produced by particle acceleration technology using a purified linear DNA fragment containing a cassette coding for the Cry3Bb1 protein, as well as a cassette coding for the selectable marker, neomycin phosphotransferase type II (NPTII). Southern blot analyses confirmed that corn event MON 863 contains one copy of the transformation cassette that was inserted at a single locus in the plant genome. No additional elements from the DNA linear fragment, linked or unlinked to intact gene cassettes, were detected in the plant genome. MON 863 does not contain any detectable plasmid backbone sequence. These data support the conclusion that only the two full-length proteins, Cry3Bb1 and NPTII, are encoded by the transgenic insert in MON 863.

Segregation analysis of the CRW-protected phenotype across five generations confirmed the heritability and stability of the *cry3Bb1* coding sequence. Southern blot analysis of DNA extracted from plants spanning three generations further confirmed the stability of the inserted genes in MON 863.

Fifty-one compositional components of corn grain and forage collected from replicated field trials were evaluated as part of a nutritional assessment of MON 863. The majority of differences between MON 863 and parental control values for each component analyzed were found to be insignificant. Twelve percent of the comparisons made were significant at the ($p<0.05$) level. All MON 863 compositional values fell within the range of values observed for commercial maize lines planted at the test sites. They also fell within historical control and literature ranges. These analyses demonstrate that the composition of grain and forage from corn event MON 863 is substantially equivalent to conventional corn varieties.

The *Bacillus thuringiensis (B.t.)* Cry proteins have a long history of safe use in agriculture. The deduced amino acid sequence of the *B.t.* protein expressed in MON 863 is >98.9% identical to that of the Cry3Bb1 protein contained in the foliar-applied commercial product, *Raven® Oil Flowable Bioinsecticide*. Based on toxicological evaluations, the Cry3Bb1 protein poses minimal risk to humans and domestic animals. Acute oral administration of Cry3Bb1 to mice was without effect at the highest dose tested of 3,200 mg/kg. *In vitro* digestive fate studies have shown that the protein is degraded to small pesticidally inactive

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fragments in a matter of seconds. Furthermore, the protein is not glycosylated and it lacks sequence similarity to known allergens and toxins.

The level of Cry3Bb1 protein in corn was determined in multiple plant tissues collected from replicated field trials. The mean levels of Cry3Bb1 protein found in grain and forage were 70 and 39 µg/g of fresh tissue weight, respectively. Based on these protein levels and upper bound estimates for corn consumption by humans, the margin of safety (MOS) for Cry3Bb1 in humans is $5x10^4$.

Agronomic, morphological and pest susceptibility observations have been recorded in multiple field trials conducted across major corn growing regions of the United States (U.S.) and Canada. Results from these trials confirm that MON 863 is phenotypically equivalent to conventional corn except for its tolerance to CRW and other coleopteran pests. Collectively, the data summarized in this document support a conclusion that food and feed products containing event MON 863 are as safe and nutritious for human and animal consumption as those derived from conventional corn.

3.1.2 Identifiers of the New GM organism

Corn Rootworm Protected Corn Event MON 863 (hereafter referred to as MON 863).

3.1.3 Brand Name of Food Derived from the New GM Organism

The brand name proposed for corn seed containing event MON 863 to be planted in the U.S. and Canada is: *YieldGard® Rootworm Corn Seed.*

3.1.4 Food Likely to Contain the New GM Organism

Although an ideal source of energy, little whole kernel or processed corn is consumed by humans in Australian and New Zealand when compared to corn-based food ingredients.

Corn is used in the manufacture of :

- corn flakes
- baking products
- extruded confectionery
- corn chips
- processed fractions such as oils and fructose syrups

The corn chip market is experiencing year-on-year growth. Corn starch is used by the food industry for the manufacture of dessert mixes and canned foods. Alternatives to corn are not readily available for these uses. The use of ethanol derived from corn starch is increasingly being used as an additive to automotive fuels (NCGA, 2002).

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3.2 HISTORY OF USE

3.2.1 Donor Organism(s)

Donor Organism: *Bacillus thuringiensis*

B.t. is a spore-forming, gram-positive bacterium that is found naturally in soil. *B.t.* produces parasporal inclusions (*i.e.*, crystals) during the stationary and sporulation phases of growth that contain proteins toxic to selected insect species. The appearance of parasporal inclusions distinguishes *B.t.* from the other common soil bacterium, *Bacillus cereus*.

Corporate, institutional and government collections of *B.t.* contain thousands of strain isolates from around the world. Several of these strain isolates have been extensively studied and commercialized as active ingredients for biopesticidal products (Baum *et al.*, 1996). These products display selective insecticidal activity against a number of pests, including: *B.t.* subsp. *israelensis* strains that are active against dipteran insects (*e.g.*, mosquitoes and black flies); *B.t.* subsp. *tenebrionis* and *kumamotoensis* strains that are active against coleopteran insects (*e.g.*, corn rootworm, Colorado potato beetle, elm leaf beetle and yellow mealworm); and *B.t.* subsp. *kurstaki*, *thuringiensis*, *sotto* and *aizawai* strains that are active against lepidopteran insects (*e.g.*, European corn borer, tomato hornworm, gypsy moth, cabbage looper, tobacco budworm and corn earworm). Biopesticidal products based on recombinant *B.t.* strains have also been commercialized for use in agriculture since the 1960's. Typically, commercial quantities of these microbes are prepared in large-scale cultures in which the bacteria are allowed to sporulate. The spores and proteins are then formulated for application to plants (Bernhard and Utz, 1993).

An exemption from the requirement of a tolerance (*i.e.*, maximum residue limit) for the first microbial *B.t.* product was granted in 1960 by the U.S. Food and Drug Administration (FDA) after an extensive toxicity and infectivity evaluation program. The testing program consisted of acute, subchronic, and chronic studies, which resembled the testing required for conventional chemical pesticides. Registration was granted by the U.S. Department of Agriculture (USDA) later that same year. In 1971, the U.S. Environmental Protection Agency (EPA) assumed responsibility for all pesticide tolerance exemptions for microbial *B.t.* products. Since then, a variety of naturally occurring and genetically modified microbial *B.t.* products have been registered and included under this tolerance exemption. EPA has established separate tolerance exemptions by amendment for various Cry proteins (*e.g.*, Cry1Ab, Cry1Ac and Cry3Aa) expressed in genetically-modified food crops (EPA 1995a, 1995b, 1995c, 1996 and 1997). The EPA recently established an exemption from the requirement of a tolerance specifically for Cry3Bb1 protein in corn commodities (EPA, 2001). The conclusion of reasonable certainty of no harm and the resultant tolerance exemptions for this wide array of *B.t.* mixtures and Cry proteins in food or feed were based on the lack of adverse effects to mammals in numerous toxicological studies. This conclusion is supported by a history of safe use in agriculture for over 40 years (McClintock *et al.*, 1995). There are no adverse effects known to have occurred in humans during this prolonged period of use (EPA, 1998).

The safety of *B.t.* as a donor organism has previously been reviewed in Monsanto consultations with ANZFA and FDA for genetically modified cotton, corn and potato

products. The characteristics of this organism do not warrant additional analytical or toxicological testing.

Donor Organism: *Escherichia coli*

The bacterium *Eschericia coli* is ubiquitous in the environment and found in the digestive tracts of vertebrate species, including humans (Jefferson *et al.*, 1986). *E. coli* strains are commonly used as protein production systems in many commercial applications (Bogosian and Kane, 1991). Safety of the donor organism, *E. coli*, has previously been assessed by FDA as part of the consultation process for other transformed crops that contain the *nptII* gene (FDA, 1998). The *nptII* gene was isolated from prokaryotic transposon Tn5 present in *E. coli*. The enzyme encoded by this gene (*i.e.*, NPTII) is used as a selectable marker in the development of genetically improved plants for agriculture.

Conclusion on Donor Organism Safety

There is long history of safe use for *B.t.*-based products in agriculture and a substantial toxicology database developed for them. *E. coli* is ubiquitous in the environment and presents minimal threat to human and animal health. A conclusion of minimal risk is reached for the donor organisms in the safety assessment of this new plant variety.

3.2.2 Host Organism(s)

Corn (*Zea mays* L.) originated in Mexico and was grown as a food crop as early as 2700 BC (Salvador, 1997). The history of corn has been studied extensively and multiple hypotheses for its origin and parentage have been advanced (Mangelsdorf, 1974). The preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988).

Corn is an extremely productive crop, yielding an average of 138 bushels per acre in the U.S. during 2001 (NCGA, 2002). Its high yield makes it one of the most economical sources of metabolizable energy for feeds, and of starch and sugar for food and industrial products. Approximately two-thirds of the corn produced in the U.S. is fed to livestock. Therefore, indirect consumption is much greater than direct consumption for humans. Further discussion on the origin and utilization of modern corn can be found in multiple documents (Benson and Pearce, 1987; Galinat, 1988; OECD, 2002; NGGA, 2002; Corn Refiners Association, 2002).

The corn germplasm that was the recipient of the transgenes in event MON 863 is a publicly available inbred line of corn, A634. This inbred line was used because it responds well to particle bombardment transformation and tissue culture regeneration. Inbred A634 was released in 1965 by the Minnesota Agricultural Experiment Station at the University of Minnesota. A634 is a synthetic stiff stalk yellow dent corn derived from B14 for the northern corn belt (Henderson, 1976). It has dark green leaves, typically matures in 111 days and requires 1485 growing degree units to flower. In the most popular hybrid, Mo17 x A634, it is used as the male parent. In the early 1980s, A634 was among the five most popular public inbreds used in U.S. hybrid corn production. It has also been widely used to develop new inbred lines.

3.2.3 Corn as a Food Source

Corn Consumption in the United States

In spite of its great value as a source of energy, little whole kernel corn is consumed by humans when compared to corn-based food ingredients (Hodge, 1982; Watson, 1988). The low price and ready availability of processed corn products has resulted in the development of large volume food and industrial uses. Corn is an excellent raw material for the manufacture of starch (Anderson and Watson, 1982). Nearly one-quarter of corn starch produced is sold as starch products, whereas three-quarters of the starch is converted to a variety of sweetener and fermentation products including high fructose corn syrup and ethanol (Watson, 1988; NCGA, 2002; Anderson and Watson, 1982; White and Pollak, 1995). Additionally, corn oil is commercially processed from the germ and accounts for approximately 9% of U.S. vegetable oil production (Orthoefer and Sinram, 1987). Each of these materials is a component of many foods including bakery and dairy goods, beverages, confections and meat products.

Animal feeding represents the largest use of corn in the U.S. with approximately two-thirds of annual production being fed to cattle, chicken and swine (Hodge, 1982; Perry, 1988; Watson, 1988). Approximately 100 million metric tonnes of corn grain are fed to livestock. Another 1.5 to 2 million tonnes of wet and dry milling by-products, primarily corn gluten meal and feed, are fed directly or in formulated feeds (Perry, 1988)

Corn Consumption in Australia and New Zealand

Corn is a relatively minor crop in Australia. Production of corn in 1989-99 is estimated to be 310,000 tonnes per annum. This is less than the 1997-98 figure of 340,300 tonnes and 22% less than production in 1996-97. New Zealand production of corn is 130,000 tonnes per annum. The total area under cultivation in Australia and New Zealand is 82,000 hectares. In Australia, 185,000 tonnes are directed to stockfeed and 35,000 tonnes exported. The remaining 90,000 tonnes are used for human consumption. These statistics do not include figures for sweet corn.

Stockfeed:	60 %
Human Consumption:	30 %
Export:	10 %

Usage of corn in New Zealand follows a similar pattern, with feed supply to chicken processors dominating.

Corn is used in the manufacture of :

- corn flakes
- baking products
- extruded confectionery
- corn chips
- processed fractions such as oils and sugar syrups

The corn chip market is experiencing year-on-year growth. Corn starch is used by the food industry for the manufacture of dessert mixes and canned foods. Alternatives to corn are not readily available for these uses.

3.3 NATURE OF THE GENETIC MODIFICATION

The development of a transgenic crop like corn event MON 863 requires that thousands of plant cells are transformed and screened for incorporation of the genes of interest. Figure 1 displays a flow chart of the macro-level steps involved in the development of corn event MON 863.

3.3.1 Transformation Method

A purified DNA fragment of the Monsanto plasmid vector, PV-ZMIR13, was used for transformation of corn to create event MON 863. Figure 2 displays a plasmid map of PV-ZMIR13. This plasmid, which contains *cry3Bb1* and *nptII* gene cassettes, was amplified in *E. coli* and purified from bacterial lysates. A linear fragment containing the gene of interest and a marker gene was prepared by digestion of the plasmid with the restriction endonuclease, *Mlu* I. This linear fragment, designated ZMIR13L, was used for transformation of corn tissue.

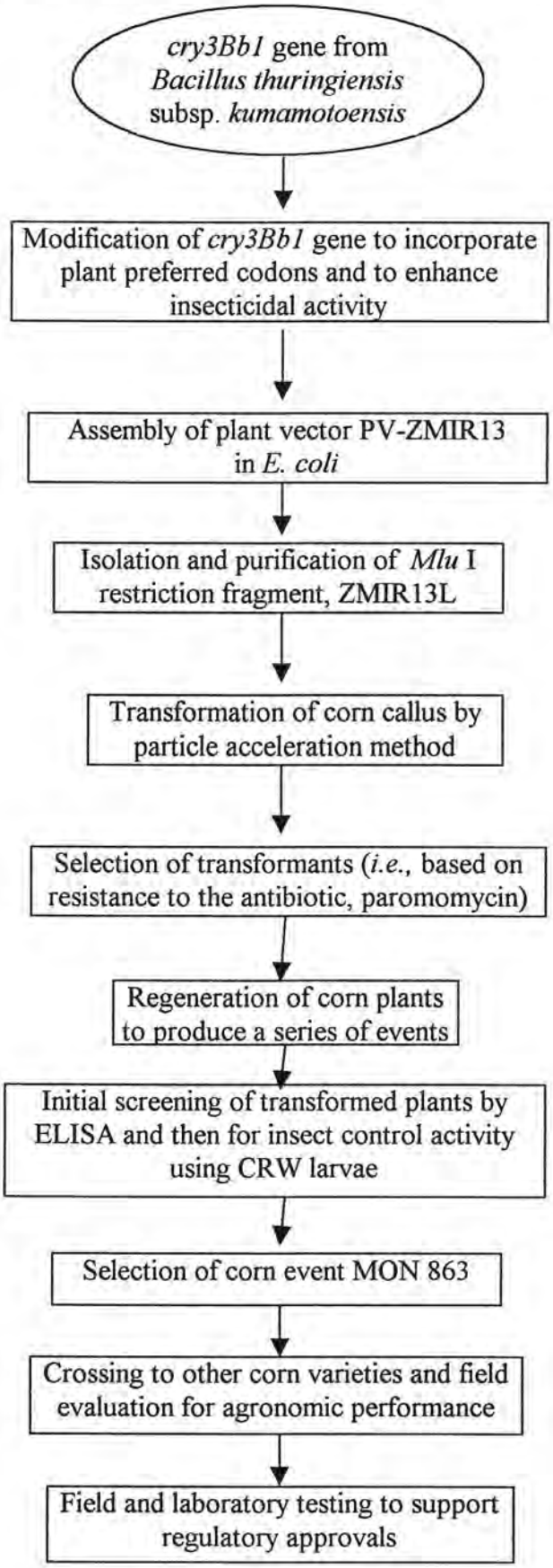
DNA was introduced into corn tissue by a particle acceleration methodology (Klein *et al.*, 1987; Gordon-Kamm *et al.*, 1990). DNA was precipitated onto microscopic tungsten or gold particles using calcium chloride and spermidine. Precipitated DNA and particles were placed onto a plastic macrocarrier and then accelerated at high velocity such that the macrocarrier was retained and particles with DNA were permitted to continue their flight with eventual penetration into the plant cells. The particle-delivered DNA was then incorporated into the plant genome. The plant cells were incubated on a tissue culture medium containing 2,4-D that supported callus growth. The introduced DNA contained nucleotide sequence encoding resistance to the antibiotic, paromomycin (*i.e.*, *nptII*). When grown in the presence of paromomycin, only genetically transformed cells continued to grow. Plants were regenerated from the tolerant callus tissue and assayed for the presence of Cry3Bb1 protein by enzyme-linked immunosorbent assay (ELISA) methods.

3.3.2 Bacteria Used Prior to Transformation

In 1991, Rupar *et al.* reported discovery of a novel *B.t.* strain (EG4691) that produced a crystal protein that displayed activity against the southern corn rootworm. Donovan *et al.* (1992) isolated and sequenced the gene encoding this crystal protein, which was designated Cry3Bb1 (GenBank Accession No. M89794). Advanced molecular techniques have been directed to the design of a gene that is codon optimized for expression in plants and that encodes a Cry3Bb1 variant with enhanced activity against CRW species. This synthetic *cry3Bb1* coding sequence was spliced into a plant transformation vector and propagated in *E. coli*.

The bacterium *E. coli* was the source of the *nptII* gene isolated from prokaryotic transposon Tn5. The enzyme encoded by this gene (*i.e.*, NPTII) is a widely used selectable marker in the development of genetically improved plants for agriculture. The bacterium *E. coli* is ubiquitous in the environment and found in the digestive tracts of vertebrate species,

Figure 1. Steps in the development of corn event MON 863.



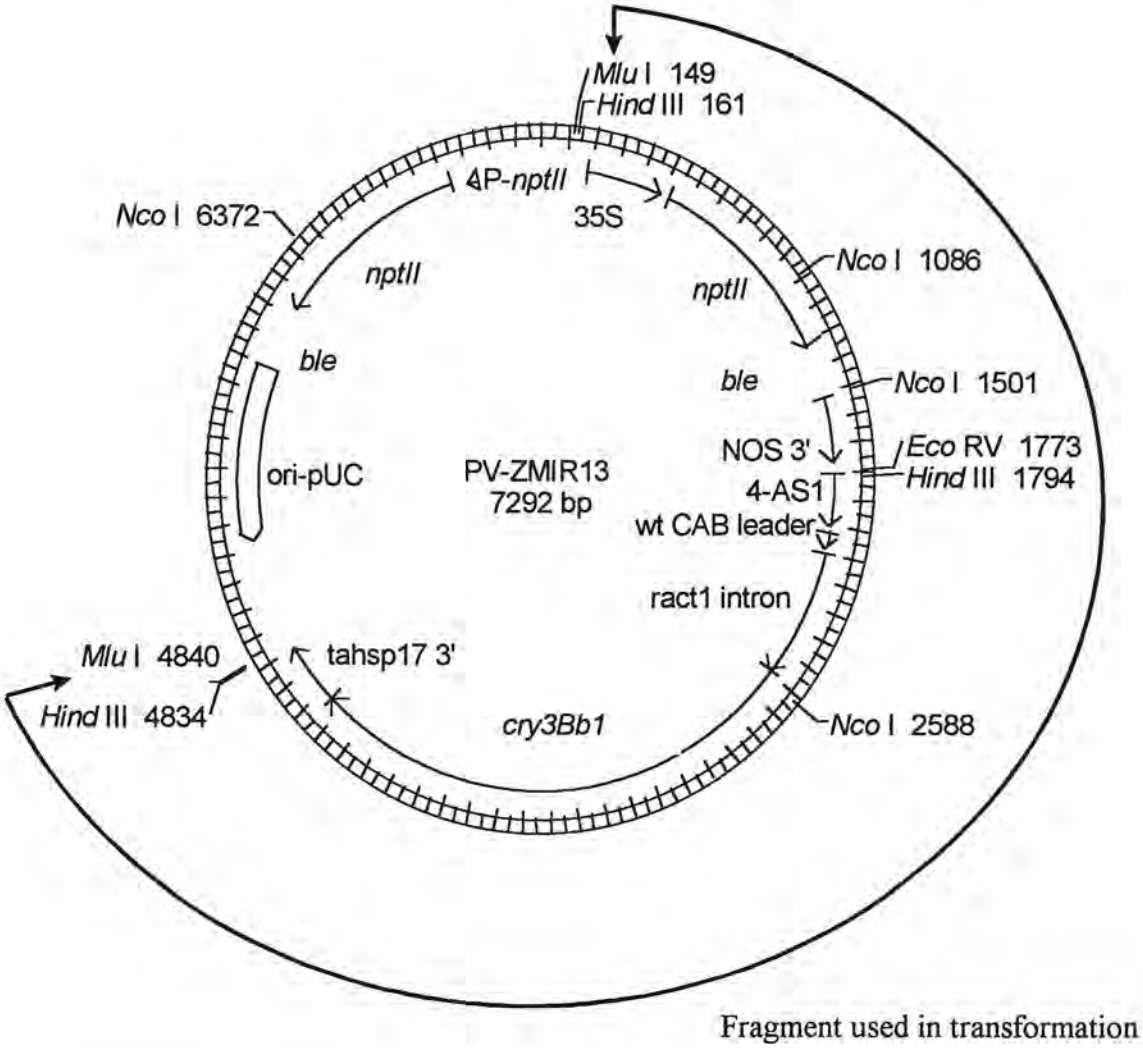
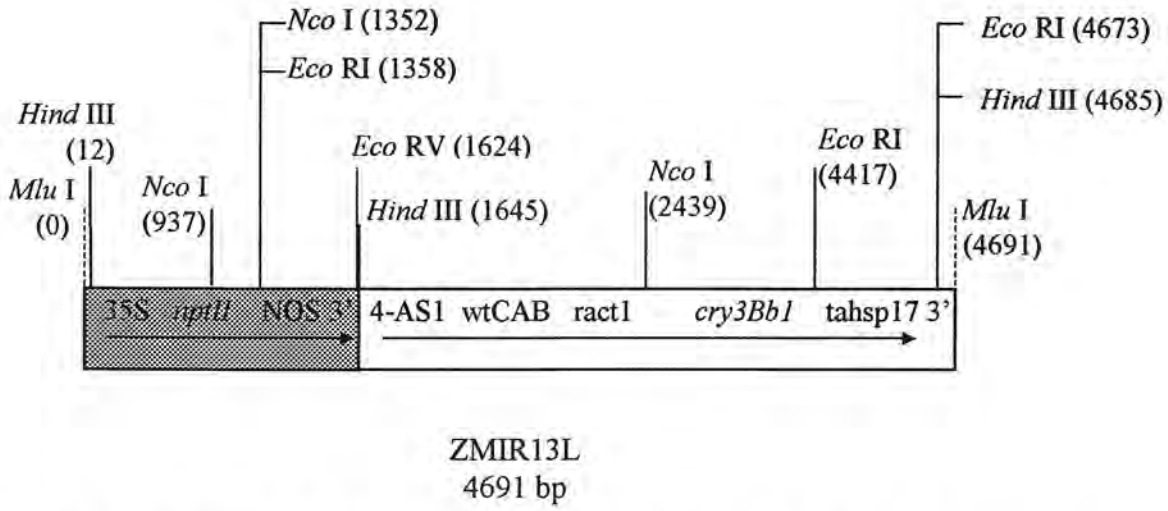


Figure 2. Plasmid map of PV-ZMIR13. Plasmid PV-ZMIR13 was the source of the *Mlu* I fragment used in the transformation of corn event MON 863.

including humans (Jefferson *et al.*, 1986). *E. coli* strains are commonly used as protein production systems in many commercial applications (Bogosian and Kane, 1991). Safety of the donor organism, *E. coli*, has previously been assessed by FDA as part of the consultation process for other transformed crops that contain the same *nptII* gene (FDA, 1998).

3.3.3 Gene Construct and Vectors

The linear vector ZMIR13L was prepared by digestion of plasmid PV-ZMIR13 with restriction endonuclease *Mlu* I. A diagrammatic representation of the linear ZMIR13L fragment is displayed in Figure 3. Plasmid backbone was separated from the *Mlu* I DNA fragment containing the *cry3Bb1* and *nptII* expression cassettes by gel electrophoresis. This *Mlu* I fragment would not be expected to contain any plasmid backbone DNA sequence except for residual DNA derived from multiple cloning sites. The *cry3Bb1* expression cassette consists of the *cry3Bb1* coding region regulated by the 4-AS1 plant promoter and the wt CAB leader, the rice actin intron, and the tahsp17 3' transcriptional termination sequence.



ZMIR13L
4691 bp

Figure 3. Linear map of DNA fragment ZMIR13L. This purified linear fragment was introduced into corn tissue by particle acceleration technology to produce transgenic event MON 863. The dashed lines represent the remaining *Mlu* I half sites following digestion of the plasmid.

The *nptII* expression cassette consists of the *nptII* coding region regulated by the 35S promoter, and the NOS 3' transcriptional termination sequence. A description of each of these elements is contained in Table 1.

Regulatory Sequences

In the *cry3Bb1* gene cassette, the *B.t.* protein coding sequence is under the control of 5' noncoding elements consisting of four repeats of activating sequence-1 (AS1; Lam and Chua, 1990) and a single portion of the 35S promoter (Odell *et al.*, 1985). The 35S promoter and AS1 are derived from cauliflower mosaic virus (CaMV). AS1 is a 21 base pair (bp) element identified from this promoter that has been associated with high levels of protein expression in roots (Lam *et al.*, 1989). The promoter sequences are followed by a 5' untranslated leader sequence from wheat chlorophyll a/b binding protein, which facilitates mRNA translation (Lamppa *et al.*, 1985), and the first intron of the rice actin 1 sequence, which enhances DNA transcription (McElroy *et al.*, 1990). All of these elements are introduced upstream of the *cry3Bb1* coding sequence. The *cry3Bb1* coding sequence is followed by a sequence from the 3' nontranslated region of the gene encoding wheat heat shock protein 17.3, which ends transcription and directs polyadenylation (McElwain and Spiker, 1989).

The selectable marker cassette contains *nptII* coding sequence under the control of a 35S CaMV promoter (Odell, *et al.*, 1985). The *nptII* gene originated from *E. coli* transposon, Tn5. Due to the use of a unique restriction site for the excision of *nptII* from Tn5, this gene cassette also contains a 153 bp portion of the 378 bp bleomycin binding protein gene (*ble*). This fragment of *ble* is located 20 nucleotides downstream of the *nptII* stop codon. It is joined to the nopaline synthase 3' nontranslated sequence, NOS 3', from *Agrobacterium tumefaciens* T-DNA, which ends transcription and directs mRNA polyadenylation (Bevan *et al.*, 1983).

Table 1. Genetic elements present in the *Mlu* I restriction fragment ZMIR13L.

Genetic Element	Size (kb)	Function
cryBb1 gene cassette:		
4-AS1	0.22	Promoter containing 4 tandem copies of AS1 and a single portion of the 35S promoter of CaMV
wt CAB	0.06	5' untranslated leader of the wheat chlorophyll a/b-binding protein
Ract 1 intron	0.49	Intron from the rice actin gene
Cry3Bb1	1.96	Coding sequence for a synthetic variant of the wild type Cry3Bb1 protein produced in *Bacillus thuringiensis* subsp. *kumamotoensis*
tahsp 17'3	0.23	3' nontranslated region of the coding sequence for wheat heat shock protein 17.3 that ends transcription and directs polyadenylation
Selectable marker elements:		
35S	0.35	CaMV promoter
nptII	0.97	Coding sequence for the enzyme NPTII from *Eschericia coli* transposon, Tn5. The DNA derived from *E. coli* also includes a 0.153 kb segment of the *ble* gene
NOS 3'	0.26	3' nontranslated region of the nopaline synthase gene of *Agrobacterium tumefacians* T-DNA that ends cell transcription and directs polyadenylation

The mRNA that is transcribed from this cassette contains tandem open reading frames (ORF). The proximal ORF is the complete *nptII* coding sequence while the distal ORF encodes approximately 40% of the bleomycin binding protein (BLE) sequence. If translated, this distal ORF would yield a 10.25 kDa protein containing amino acids 1-51 of the BLE protein linked to four amino acid residues encoded by a DNA cloning linker, followed by 34 amino acid residues encoded by the NOS 3' transcriptional terminator. Although the *nptII* transcriptional unit could theoretically yield an mRNA that contains both *nptII* and *ble* 10.25 kDa coding sequences, it is predicted that only the *nptII* coding sequence will be transcribed in plants. This prediction is based on a substantial body of published data which demonstrates that eukaryotic translational initiation occurs via a ribosomal mRNA scanning mechanism (Hinnebusch, 1997; Kozak, 1987). In eukaryotes, ribosomal subunits identify a unique structure at the 5' end of the mRNA called a 'cap'. Once bound to the 5' end of the mRNA, a ribosome scans the mRNA until the first contextually correct AUG start codon is identified and translation is initiated. Translation then continues until a stop codon is encountered. Three mechanisms that facilitate the translation of more than one ORF in eukaryotic polycistronic mRNA (*i.e.*, mRNAs that contain two or more non-overlapping

ORFs) have been described. Two of the translational mechanisms are entirely dependent upon the size and positioning of the ORFs within the mRNA.

The first translational mechanism involves ribosome frame shifting or the bypass of the stop codon in the 5' ORF such that translation yields a polypeptide composed of both ORFs. The second translational mechanism involves translational reinitiation at a second ORF whose start codon is located downstream of the 5' ORF stop codon. The final mechanism involves the presence of an internal ribosome entry site in the mRNA that permits bypass of the 5' cap. Examination of the *nptII* transcriptional unit mRNA sequence shows that neither the size of the *ble 10.25* coding sequence, nor positioning relative to the NPTII ORF would facilitate its translation. Moreover, the distance separating the NPTII stop codon and the BLE 10.25 start codon is insufficient to contain an internal ribosome entry site. Given these constraints, it is predicted that only NPTII will be translated from the mRNA expressed by the *nptII* transcriptional unit.

A western blot analysis confirmed that BLE 10.25 is not produced in the grain of corn event MON 863 (Silvanovich *et al.*, 2001). Rabbit antiserum that is capable of detecting the full-length BLE protein was used in this study to demonstrate the absence of detectable plant-produced BLE 10.25, while *E. coli*-produced BLE 10.25, identical to the plant putative BLE 10.25, was used as the positive control. Through spiking experiments, it was also shown that corn grain extracts do not mask the immunoreactivity of *E. coli*-produced BLE 10.25. Using western blot analysis, it was determined that BLE 10.25 is not detectable in grain from corn event MON 863 above the limit of detection of 1.7 ppm. These data corroborate the prediction that BLE 10.25 protein is not produced in grain from corn event MON 863.

The *cry3Bb1* Coding Sequence

Event MON 863 contains a *cry3Bb1* coding cassette. This cassette encodes a Cry3Bb1 variant with enhanced insecticidal activity against CRW species. The *cry3Bb1* gene has been codon optimized for expression in monocotyledonous plants. To facilitate linkage of this *cry3Bb1* gene to a plant-effective promoter it was necessary to create a restriction endonuclease site at the 5' end of the nucleotide coding sequence. This was accomplished by insertion of the nucleotides 'GCC' at positions 4, 5 and 6 of the coding sequence. Insertion of this *Nco* I restriction site had the effect of introducing an alanine residue at position two of the encoded protein.

The *nptII* Coding Sequence

Event MON 863 contains an *nptII* coding cassette. The *nptII* gene originated from *E. coli* transposon Tn5 (Beck *et al.*, 1982). NPTII functions as a dominant selectable marker in the initial laboratory stages of plant cell selection following transformation (Horsch *et al.*, 1984; DeBlock *et al.*, 1984).

3.3.4 Molecular Characterization of the Genetic Modification

Molecular analysis was performed to characterize the DNA inserted into corn to produce event MON 863. Genomic DNA was analyzed for the number of insertion sites in the plant genome; the copy number of the inserted DNA; the integrity of the inserted promoters, coding regions, and terminators; and the presence of plasmid backbone sequence. DNA

extracted from event MON 863 tissue was digested with a variety of restriction endonucleases and subjected to Southern blot hybridization analysis. Control genomic DNA was digested with the same restriction enzymes as used for MON 863. Digested DNA was separated by means of agarose gel electrophoresis. Long runs were used for separation of high molecular weight DNA fragments. Short runs were used to ensure that all DNA restriction fragments were retained on the gel. The locations of the restriction sites utilized for Southern analyses are shown in the plasmid map in Figure 2 and the linear DNA fragment map of ZMIR13L in Figure 3.

Polymerase chain reactions (PCR) were performed to verify the 5' and 3' insert-to-plant junctions, as well as to determine whether the 5' and 3' ends of the insert were intact. A detailed discussion of the materials, methods and results for these molecular characterization can be found in the report prepared by Cavato *et al.* (2001). Nontransgenic corn of comparable germplasm served as a control for the molecular characterization, protein expression and compositional analysis studies.

Southern blot analyses confirmed that event MON 863 contains one DNA insert located on a 5.0 kb *Nde* I restriction fragment. This insert contains one copy of the *Mlu* I plasmid fragment used in transformation. No additional elements from the DNA fragment used in transformation, linked or unlinked to intact cassettes, were detected in the genome. A summary of the molecular findings is presented in Table 2.

Table 2. Summary of molecular characterization findings for corn event MON 863.

Genetic Element	Findings for MON 863
# of transgene insertions	1
# of copies of *cry3Bb1* cassette	1
# of copies of *nptII* cassette	1
4-AS1 + wt CAB + ract 1	Intact
cry3Bb1 coding sequence	Intact
tahsp17 3' transcriptional terminator	Intact
35S promoter	Intact
nptII coding sequence	Intact
NOS 3' transcriptional terminator	Intact
Plasmid backbone	None

PCR and DNA sequencing were used to verify the 5' and 3' junction sequences of the insert with the plant genome, as well as the intactness of the 5' and 3' ends of the insert. Approximately 10 bp from the 3' end of ZMIR13L, including the *Hind* III restriction site, are missing. However, the tahsp17 3' transcription termination sequence is intact. Additionally, event MON 863 does not contain any detectable plasmid backbone sequence, including *ori*-pUC or the *nptII* coding region regulated by a bacterial promoter. These data support the conclusion that only the two full-length proteins, Cry3Bb1 and NPTII, should be encoded by the insert in event MON 863. A schematic representation of the inserted DNA in MON 863 is displayed in Figure 4.

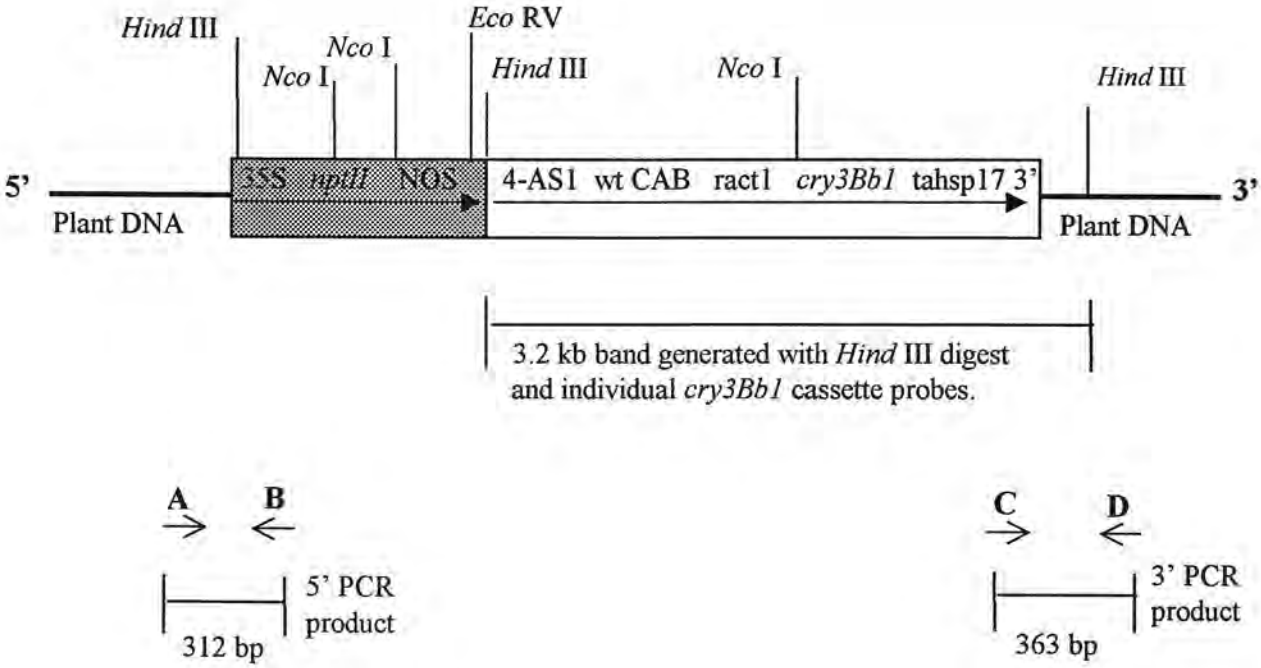


Figure 4. Schematic representation of the MON 863 insert. This figure depicts the predicted insert in corn event MON 863 based on data from Southern blot analyses and PCR confirmation of the sequences at the 5' and 3' ends of the insert. There is one copy of the ZMIR13L fragment that was used to generate corn rootworm event MON 863. Genomic flanking sequence on the 3' end indicates that approximately 10 bp, including the *Hind* III site and *Mlu* I half site, are missing. However, the tahsp17 3' transcription termination sequence is intact.

Study reports being submitted by Monsanto in support of this application include:

Cavato, T. A., E. C. Rigden, D. W. Mittanck and R. P. Lirette (2001). Amended Report for MSL-16505: Molecular analysis of corn event MON 863. MSL-17152, an unpublished study conducted by Monsanto Company.

Silvanovich, A., K. Karunanandaa, R. S. Thoma, J. Blasberg and J. D. Astwood (2001). The absence of detectable ble translation products in corn grain containing event MON 863. MSL-17449, an unpublished study conducted by Monsanto Company.

3.3.5 Segregation and Stability of Gene Transfer

Chi square analysis of Mendelian inheritance data over five generations was performed to determine the heritability and stability of the *cry3Bb1* gene in corn varieties containing event MON 863. Expected and observed segregation frequencies of MON 863 progeny that are positive for the CRW-protected phenotype are presented in Table 3.

Plants were identified as being positive for the CRW-protected phenotype based on the presence of Cry3Bb1 protein as determined by ELISA. The $A1F_1$ generation was derived

Table 3. Comparison of expected and observed segregation frequencies for MON 863 progeny. (+) denotes plants that tested positive for the Cry3Bb1 protein; (-) denotes plants that tested negative for Cry3Bb1.

	Observed		Expected		
Generation	+	-	+	-	χ^2
$A1F_1$	41	36	38.5	38.5	0.21†
$A1F_2$	89	23	84	28	0.96†
$A1BC_1F_1$	18	15	16.5	16.5	0.12†
$A1BC_2F_1$	931	1040	985.5	985.5	5.92*
$A1BC_2F_2$	322	110	324	108	0.03†

† - Not significant at $p \leq 0.05$ (Chi square = 3.84, 1 df)
* - Significant at $p \leq 0.05$ (Chi square = 3.84, 1 df)
No significant differences observed at $p \leq 0.01$ (Chi square = 6.63)

from cross-pollinating the original transformed plant with the inbred line A1. The $A1F_2$ generation was derived from self-pollinating individual $A1F_1$ plants. The $A1BC_1F_1$ generation was derived from back-crossing $A1F_1$ plants to the nontransgenic inbred line A1. The $A1BC_2F_1$ generation was derived from back-crossing $A1BC_1F_1$ plants to the nontransgenic inbred line A1. The $A1BC_2F_2$ generation was derived from self-pollinating individual $A1BC_2F_1$ plants. Figure 5 diagrammatically presents the breeding history for MON 863 and indicates the generations used for segregation, Southern blot and expression analyses.

Genotype frequencies were compared by means of a Chi square test (Little and Hills, 1978). The Chi square value (χ^2) was computed as follows:

$$\chi^2 = \Sigma [(|o - e| - 0.5)^2 / e]$$

Where, o = observed frequency of the genotype; e = expected frequency of the genotype; and 0.5 = Yates correction factor for analysis with one degree of freedom (df).

With only one exception, all χ^2 values were less than the critical value of 3.84, thus indicating no significant differences between observed and expected frequencies for the CRW-protected phenotype across five generations of MON 863. The unusual results obtained for the $A1BC_2F_1$ generation are most likely attributable to false negatives. At the time $A1BC_2F_1$ trials were being conducted, Monsanto field researchers were experiencing about a 10% failure rate of GeneCheck® lateral flow ELISA kits used for detection of Cry3Bb1 protein in the field. The fact that a difference between observed and expected frequencies of the trait was not seen in the prior or subsequent generations (*i.e.*, $A1BC_1F_1$ and $A1BC_2F_2$) provides further evidence that the $A1BC_2F_1$ results represent an anomaly. The results of this analysis are consistent with the finding of a single active site of insertion of the *cry3Bb1* gene that segregates according to Mendel's laws of genetics. The stability of the insert has been demonstrated through three generations of cross-fertilization and two generations of self-pollination.

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MON 863 Breeding History

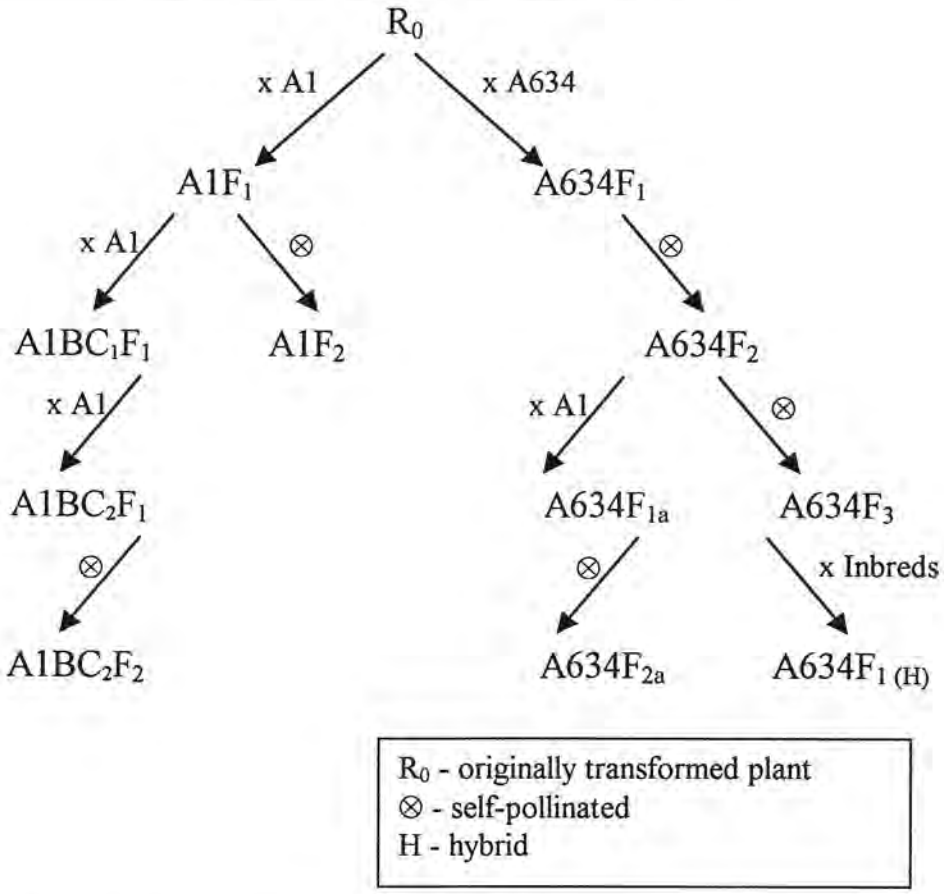

Figure 5. Breeding history for event MON 863. Segregation analysis was performed on generations $A1F_1$, $A1F_2$, $A1BC_1F_1$, $A1BC_2F_1$ and $A1BC_2F_2$. Molecular stability was performed on generations $A1F_1$, $A1F_2$, $A1BC_1F_1$, $A1BC_2F_2$, $A634F_2$ and $A634F_{2a}$. Molecular characterization was performed on generation $A634F_{2a}$. Gene expression and compositional analyses were performed on generations $A634F_{1a}$ and $A634F_{2a}$.

Southern blot fingerprint analysis of DNA extracted from plants spanning multiple generations was also conducted to evaluate the stability of the inserted DNA in corn event MON 863 (Hillyard *et al.*, 2000). Genomic DNA was extracted from grain tissue of the $A634F_2$ and $A634F_{2a}$ generations. The DNA was digested with *Nco* I, blotted, and probed with the full-length ^{32}P-labelled *nptII* coding region. Grain from nontransgenic corn served as a negative control.

Figure 6 displays the Southern blot result for generations $A634F_2$ and $A634F_{2a}$. The breeding history of these generations can be found in Figure 5. MON 846 control DNA did not show any hybridization bands, as expected for a negative control (lane 1). Control DNA spiked with PV-ZMIR13 DNA showed bands at approximately 0.4, 2.0 and 3.7 kb, which are consistent with the expected sizes of fragments following digestion of the plasmid with *Nco* I (lane 2). Two additional faint bands are observed at approximately 2.4 kb and 6.4 kb; these fragments are most likely attributable to partial digestion of the plasmid. MON 863 DNA from generations $A634F_2$ and $A634F_{2a}$ generated bands at approximately 0.4 and 8.0 kb,

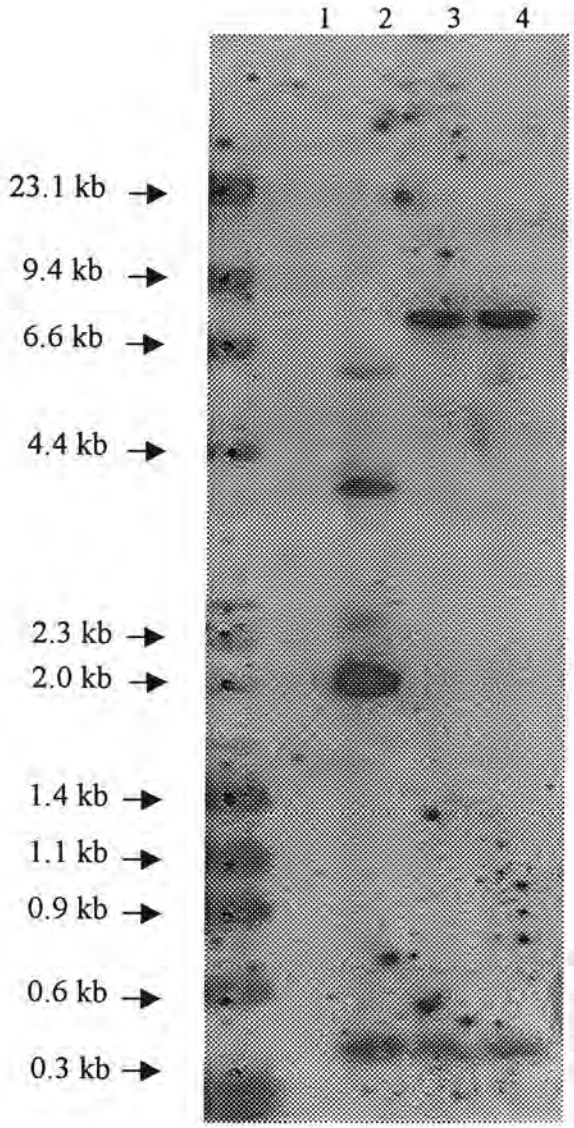

Figure 6. Southern blot analysis of event MON 863: Stability of inserted DNA. Control and MON 863 genomic DNA extracted from grain and seed were digested with *Nco* I. The blot was probed with a ^{32}P-labeled full-length *nptII* coding region. Lane designations are as follows:

Lane 1: Control grain DNA [10 µg]
2: Control grain DNA [10 µg] spiked with ~19 pg PV-ZMIR13 (1.0 copy)
3: MON 863 A634F_2 generation grain DNA [10 µg]
4: MON 863 A634F_{2a} generation grain DNA [10 µg]

→ Symbol denotes sizes obtained from MW markers on ethidium stained gel.

which are consistent with the expected sizes of fragments resulting from a *Nco* I digest of the cassettes inserted in MON 863 (lanes 3 & 4). The band at 0.4 kb represents an internal segment of the insert while the band at 8.0 kb represents the insert fragment containing *nptII* plus genomic DNA off the 5' end of the insert. No differences in banding pattern were observed for DNA extracted from the two generations tested. These results demonstrate the stability of the inserted DNA in MON 863 across multiple generations.

Studies being submitted by Monsanto in support of this application include:

Hillyard, J. R., M. Y. Deng, T. A. Cavato and R. P. Lirette (2000). Molecular analysis to determine the genetic stability of corn rootworm event MON 863 across multiple generations. MSL-17063, an unpublished study conducted by Monsanto Company.

3.4 ANTIBIOTIC RESISTANCE GENES

3.4.1 Importance of Respective Antibiotic(s)

The use of neomycin and related aminoglycoside antibiotics in Australia and New Zealand is relatively rare and resistance to these pharmaceuticals is already widespread.

3.4.2 GM Microorganism in Food

The new genetically modified organism, corn rootworm protected corn event MON 863, is not a microorgansim and does not contain whole, viable microorganisms.

3.4.3 DNA from GM Organism in Food

It is expected that DNA could be present in the following components of corn:

- starch (native)
- modified starch ingredients
- meal/semolina
- flour

Conversely, DNA is not expected in the following processed fractions:

- refined oils
- glucose syrups
- high fructose corn syrup
- maltodextrins
- dextrose

3.5 CHARACTERIZATION OF NOVEL PROTEINS

3.5.1 Function of Novel Proteins

<u>Cry3Bb1 Protein</u>

B.t. is a spore-forming, gram-positive bacterium that is found naturally in soil. *B.t.* produces parasporal inclusions (*i.e.*, crystals) during the stationary and sporulation phases of growth that contain proteins toxic to selected insect species. The proteins contained within the

parasporal inclusions, known as Cry proteins or δ-endotoxins, comprise a diverse group of insecticidal agents. Cry proteins with toxicity towards lepidopteran, dipteran, homopteran, hemipteran, and coleopteran insect larvae have been well documented. Proteins with toxicity towards nematodes, protozoans, flatworms, and mites have also been reported (Feitelson *et al.*, 1992; Feitelson, 1993). The Cry proteins are classified on the basis of amino acid sequence identity using recently adopted standardized nomenclature (Crickmore *et al.*, 1998). Cry proteins with the same Arabic numeral, which defines a primary class (*e.g.*, Cry1), share at least 45 percent amino acid sequence homology. Those with the same Arabic numeral and upper case letter (*e.g.*, Cry1A) share at least 75 percent sequence homology. The same Arabic numeral and upper and lower case letter (*e.g.*, Cry1Aa) designate greater than 95 percent sequence homology.

The *B.t.* Cry proteins comprise at least four distinct protein families that have co-evolved toxicity towards different orders of insects. Presently, there are at least 32 primary classes of Cry proteins (Cry1 – Cry32) and two primary classes of cytolytic, or Cyt, proteins (Cyt1, Cyt2), ranging in size from 25 kDa to over 130 kDa in molecular mass. These proteins vary widely in their toxicity towards insect species. Thus, variations in amino acid sequence, even within a primary Cry protein class, can lead to structural differences that translate into varying insecticidal activities.

A review of the research characterizing the mechanism of action for *B.t.* crystal proteins has been published by Schnepf *et al.* (1998). Based on the accumulated knowledge of *B.t.* Cry proteins, a generalized mode of action has been proposed and includes the following steps: ingestion of crystals by the insect, solubilization of the crystals in the insect midgut, proteolytic processing of the released Cry protein by digestive enzymes to activate the toxin, binding of the toxin to receptors on the surface of midgut epithelial cells, formation of membrane ion channels or pores, and consequent disruption of cellular homeostasis (English and Slatin, 1992). Electrolyte imbalance and pH changes render the gut paralyzed, which causes the insect to stop eating and die (Sacchi *et al.*, 1986).

The insecticidal specificity of a Cry protein can be determined by any number of steps in its mechanism of action, including solubilization of the crystal, proteolytic processing and stability of the toxin, receptor affinity, and the formation of ion channels and pores within the membrane. Receptor binding, in particular, is a critical step in the mechanism of action of the Cry proteins because, without it, no toxic effect can be exerted. Irreversible binding of toxins to midgut receptors appears to be correlated with insect susceptibility. This is a key factor in assessing the safety of Cry proteins for nontarget organisms such as fish, birds and mammals. No receptors for these proteins have been identified on intestinal cells of mammals to date (Sacchi *et al.*, 1986; Noteborn, 1994; Van Mellaert *et al.*, 1988). This explains, in part, the absence of toxicity for the protein δ-endotoxins of *B.t.* subspecies such as *kurstaki* to nontarget organisms.

In 1991, Rupar *et al.* reported discovery of a novel *B.t.* strain (EG4691) that produced a crystal protein with insecticidal activity against the southern corn rootworm (*Diabrotica undecimpunctata howardi*). Donovan *et al.* (1992) isolated and sequenced the gene encoding this crystal protein, which was designated as CryIIIB2. Following the adoption of standardized nomenclature for identifying *B.t.* crystal proteins, the protein isolated from strain EG4691 was renamed Cry3Bb1.

Recently developed molecular techniques have been directed to the design of genes that encode proteins with enhanced insecticidal activity. English *et al.*, (2000) have designed multiple genes encoding Cry3Bb1 variants with enhanced activity against CRW species. These variants are virtually identical in structure to the Cry3Bb1 wild type protein with the exception of a small number of strategically placed amino acid substitutions that impact insecticidal activity. The Cry3Bb1 variant produced in corn event MON 863 is approximately eight times more lethal to southern corn rootworm larvae than the wild type protein.

NPTII protein

Event MON 863 contains the selectable marker, neomycin phosphotransferase type II. NPTII functions as a dominant selectable marker in the initial laboratory stages of plant cell selection following transformation. The NPTII enzyme encoded by the *nptII* cassette uses ATP to phosphorylate neomycin and related aminoglycoside antibiotics, thereby inactivating them. Cells that produce the NPTII enzyme selectively survive exposure to these aminoglycosides. The purpose of inserting *nptII* into corn cells, along with the *cry3Bb1* cassette, is to have an effective method for selecting cells that contain the inserted genes and that can be used in bacterial selection during construction of the plasmid. In general, the frequency of cells that are transformed is often low, ranging from $1x10^{-4}$ or $1x10^{-5}$ of cells treated (Fraley *et al.*, 1983). Therefore, the selectable marker, *nptII*, and the selection agent, paromomycin, are used to facilitate the screening process.

3.5.2 Level and Site of Novel Protein Expression

Validated ELISA methods were used to estimate the levels of Cry3Bb1 and NPTII proteins in tissues of MON 863 and nontransgenic corn (Dudin *et al.*, 2001). MON 863 seed from generation $A634F_{1a}$ was planted in order to produce tissues for this analysis. Levels of Cry3Bb1 and NPTII proteins were determined in tissues collected from MON 863 plants grown under field conditions at multiple sites. Tissue samples from nontransgenic plants of comparable germplasm (MON 846) served as controls and were analyzed for the presence of both proteins.

Tissue samples were collected from plants grown in four U.S. field trials conducted in Iowa (two sites), Nebraska, and Illinois during the 1999 growing season. Three additional sites in Argentina were used for harvesting of pollen during the winter of 2000. Collectively these sites provided a variety of environmental conditions representative of regions where corn rootworm protected corn lines would be grown as commercial products. MON 863 and MON 846 were planted in four replicate plots at each location.

Composite samples of young leaf (V4 stage), forage, mature root and grain were collected from each replicate at the four U.S. sites; only one replicate from each site was analyzed. At three of the U.S. sites, single plot composite samples of leaf, whole plant and root were collected throughout the growing season and evaluated. A composite sample of silk was evaluated from one U.S. site. Composite samples of pollen were evaluated from one U.S. site and from twelve plots planted at three sites in Argentina. Cry3Bb1 protein levels were measured in all tissues. NPTII protein levels were evaluated only in samples of young leaf, forage and grain taken from all four sites.

Cry3Bb1 and NPTII values are expressed as micrograms (µg) of expressed protein per gram (g) of tissue on a fresh weight (fw) basis. All values have been corrected for assay bias as determined during the method validation. The identity of MON 863 tissue samples collected from all sites was confirmed throughout various stages of this investigation by an event-specific PCR assay. Molecular analysis also confirmed the expected absence of *cry3Bb1* and *nptII* coding sequences in the control plants.

Table 4 presents a summary of Cry3Bb1 and NPTII protein levels in MON 863 tissues collected from multiple sites. Mean levels of Cry3Bb1 protein in MON 863 plants were 81 µg/g in young leaf, 70 µg/g in grain, 41 µg/g in root, and 39 µg/g in forage tissues. Cry3Bb1 levels in pollen and silk were 62 and 10 µg/g, respectively. NPTII protein levels in all tissues tested ranged from nondetectable (<0.076 µg/g) to 1.4 µg/g.

Table 4. Summary of Cry3Bb1 and NPTII protein levels measured in MON 863 tissue samples collected from multiple field sites.

Tissue (days post-planting)	Parameter*	Cry3Bb1 (µg/g fw)	NPTII (µg/g fw)
Young Leaf (21 days)	Mean ± SD Range (n)	81 ± 11 65 – 93 (4)	0.98 ± 0.27 0.74 – 1.4 (4)
Forage (90 days)	Mean ± SD Range (n)	39 ± 10 24 – 45 (4)	0.19 ± 0.03 0.17 – 0.23 (4)
Mature Root (90 days)	Mean ± SD Range (n)	41 ± 13 25 – 56 (4)	Not Analyzed
Grain (125 days)	Mean ± SD Range (n)	70 ± 17 49 – 86 (4)	<0.076† n/a (4)
Silk (58 days)	Mean ± SD (n)	10 n/a (1)	Not Analyzed
Pollen (60 days)	Mean ± SD Range (n)	62 ± 18 30 – 93 (13)	Not Analyzed

* SD = standard deviation of the mean; n = number of replicates analyzed
† Limit of detection for corn grain = 0.076 µg/g fw

Table 5 presents a summary of Cry3Bb1 protein levels in selected MON 863 tissues sampled over the course of a growing season. Cry3Bb1 and NPTII levels were below the limit of detection in control plant tissues and thus are not reported. Mean levels of Cry3Bb1 protein declined during the growing season in leaf tissue, whole plant and root tissue of MON 863. Mean levels in root tissue ranged from a high of 58 µg/g in young plants to a low of 24 µg/g in senescent plants. Cry3Bb1 protein levels in root tissue were sufficient to confer protection from CRW feeding damage during the critical early periods of plant development.

Table 5. Summary of Cry3Bb1 protein levels measured in MON 863 tissue samples collected over the 1999 growing season.

Days Post-planting	Parameter [a]	Cry3Bb1 in Leaf (µg/g fw)	Cry3Bb1 in Whole Plant [b] (µg/g fw)	Cry3Bb1 in Root (µg/g fw)
21 days	Mean ± SD Range (n)	81 ± 14 65 – 93 (3)	NC†	NC
35 days	Mean ± SD Range (n)	79 ± 6.4 72 – 84 (3)	46 ± 7.8 38 – 54 (3)	58 ± 10 46 – 66 (3)
49 days	Mean ± SD Range (n)	43 ± 18 30 – 56 (2)	31 ± 3.3 28 – 33 (2)	57 ± 3.8 54 – 59 (2)
90 days	Mean ± SD Range (n)	NC	37 ± 12 24 – 45 (3)	37 ± 11 25 – 47 (3)
126 days	Mean ± SD Range (n)	NC	25 ± 11 13 – 35 (3)	24 ± 18 3.2 – 36 (3)

a SD = standard deviation of the mean; n = number of replicates analyzed
b Only the above ground portion of the plant was included in these samples
† NC = not collected

CRW species that colonize U.S. corn fields develop through a single generation over the growing season. They overwinter as eggs with larvae emerging in the late spring to coincide with corn plantings. The timing of egg hatch is dependent upon soil temperature. CRW larvae develop through three instars, transform into inactive pupae and then emerge as adults. Adults mate and lay eggs in the fall. The elaspsed time from egg hatch to adult emergence ranges from 23-46 days and is influenced by temperature (Sutter, 1999). The pupal stage typically lasts 6-13 days, during which time the CRW do not feed. Except under the most unusual circumstances, it is highly unlikely that CRW would still be in the larval feeding stage when Cry3Bb1 levels in root begin to decline. As noted in Table 5, Cry3Bb1 protein levels are seen to decline after 49 days post-planting.

Study reports being submitted by Monsanto in support of this application include:

Dudin, Y.A., B. P. Tonnu, L. D. Albee and R. P. Lirette (2001). Amended report for MSL-16559: *B.t.* Cry3Bb1.11098 and NPTII Protein Levels in Tissue Collected from Corn Event MON 863 Grown in 1999 Field Trials. MSL-17181, an unpublished study conducted by Monsanto Company.

3.5.3 Nonexpressed Genes

The *nptII* gene cassette contains a portion of the *ble* gene. For reasons discussed in Section 3.3.3 this gene fragment is not expressed in corn event MON 863.

3.5.4 Prior History of Novel Protein Consumption

B.t. Cry Proteins

The *B.t.* proteins within the Cry3 class have been registered and exempted from the requirement of a tolerance by EPA, specifically, Cry3Aa4 and Cry3Bb1. Cry3Aa4 has been commercialized in the U. S. and other countries both as a microbial-derived *B.t.* mixture, *Foil® Biological Insecticide*, and when expressed in genetically modified plants to control Colorado potato beetle in potatoes (Perlak *et al.*, 1993). Cry3Bb1, one of the active ingredients in the microbial pesticide mixture *Raven Oil Flowable Bioinsecticide*, was commercialized in the U.S. in 1995 to control coleopteran pests in various crops (Baum *et al.*, 1996).

NPTII Protein

A history of safe consumption for NPTII exists since it has been present as a selectable marker in commercial transgenic tomato, potato, cotton crops and oilseed rape crops. The *nptII* gene is ubiquitous in the environmental by virtue of its presence in *E. coli*.

3.5.5 Acute Toxicity

Cry3Bb1 Protein

B.t. has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). The extremely low mammalian toxicity of *B.t.*-based insecticide products has been demonstrated in numerous safety studies (McClintock *et al.*, 1995). The results of studies filed in support of this application, as well as the results of studies conducted with other Cry3 proteins, demonstrate that this class of proteins is essentially nontoxic to mammals.

Data requirements for *B.t.* proteins produced in genetically modified crops include acute oral toxicity and *in vitro* digestibility studies. These requirements are based on the fact that oral ingestion is the predominant route of exposure for humans to Cry proteins in genetically improved crops. Furthermore, when proteins are toxic they are known to act *via* acute mechanisms and generally at very low dose levels (Sjoblad *et al.*, 1992). The results of rodent acute oral toxicity tests conducted with Cry3 proteins, including multiple Cry3Bb1 variants, are summarized in Table 6. In each rodent bioassay the highest achievable dose level failed to produce evidence of treatment related adverse effects and was, therefore, considered to be a no observable effect level (NOEL).

Cry3Bb1 protein extracted and purified from a heterologous *E. coli* fermentation system was used as the test material for the acute oral toxicity study with the MON 863 variant. The identical *cry3Bb1* coding sequence used in construction of vector PV-ZMIR13 was used in the transformation of *E. coli*. Equivalence of the plant and *E. coli*-produced proteins was assessed by a comparison of results obtained from multiple analytical methods, including matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, N-terminal sequencing, immunoblotting, insect bioassay, gel electrophoresis, glycosylation

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Table 6. Acute oral NOELs for various *B.t.* and plant-produced Cry proteins.

Cry3 Protein	NOEL (mg/kg) [a]	Reference
Cry3Bb1 – MON 863 variant	≥ 3200	Bonnette and Pyla, 2001
Cry3Bb1 – Wild type present in *Raven Oil Flowable Bioinsecticide* [b]	≥ 30	Baum *et al.*, 1996
Cry3Aa4 - *NewLeaf® Potato*	≥ 5000	Lavrik *et al.*, 1995

a - NOEL in rodent acute gavage study. In all instances, the highest dose tested was the NOEL.
b - *Raven* contains a mixture of Cry3Bb1, Cry3Aa4 and CrylAc proteins. In the batch tested, Cry3 proteins constituted 40% (w/w) active ingredient; Cry3Bbl protein represents 66-75% of the Cry3 proteins present in *Raven*. The highest dose tested was 10^8 CFU/rat, which is approximately a 100 mg/kg body weight dose of total active ingredients.

analysis, and amino acid compositional analyses (Hileman *et al.*, 2001a; Thoma *et al.*, 2001). The Cry3Bb1 protein purified from this fermentation system was found to be physicochemically and functionally equivalent to the protein produced in MON 863 corn. In the acute oral toxicity study performed with laboratory mice, no mortality or grossly observable adverse effects were noted (Bonnette and Pyla, 2001). The NOEL was determined to be ≥3,200 mg/kg, which was the highest dose tested.

High-dose acute exposure studies are considered appropriate for assessing the potential toxicity of Cry proteins to mammals. EPA scientists have stated that "if toxic - proteins are known to act through acute mechanisms. Also, laboratory animals show acute toxic effects from exposure to proteins known to be toxic to humans" (Sjoblad *et al.*, 1992). The potential for human exposure to Cry3Bb1 will occur through consumption of corn or corn products containing the protein. Exposure *via* the dermal or inhalation route is unlikely since the protein is contained within the plant tissue. Thus, there appears to be little opportunity for occupational exposure to the active ingredient. The protein has been found to be short-lived in soil, thus runoff to drinking water sources will be negligible. A margin of safety (MOS) for Cry3Bb1 protein has been computed based on estimates of corn dietary intake. The MOS is defined as the ratio of an appropriate NOEL to an estimate of human daily dietary exposure (DDE). An upper bound estimate (90^{th} percentile) of human daily corn consumption has been obtained from the Exposure-1 Chronic Dietary Exposure Analysis Program[1]. The estimate of daily human corn consumption is 0.78 g corn per kg body weight and the highest level of Cry3Bb1 found in samples of corn grain is 0.086 mg protein/g of grain.

The MOS is computed as follows:

Daily corn consumption (g/kg) x Cry3Bb1 grain concentration (mg/g) = DDE (mg/kg)
NOEL (mg.kg) ÷ DDE (mg/kg) = **MOS**

Computation of the MOS for Cry3Bb1 in MON 863 corn is as follows:

0.78 g/kg x 0.086 mg/g = 0.067 mg/kg

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[1] Technical Assessment Systems, Inc.

$$3{,}200 \text{ mg/kg} \div 0.067 \text{ mg/kg} = \mathbf{4.8 \times 10^4}$$

A MOS of ≥100 is generally regarded as being protective of human health. There is a four order of magnitude difference between the acute oral NOEL and the upper bound estimate of human dietary exposure to Cry3Bb1 in MON 863. This large margin of safety ensures a reasonable certainty of no harm for humans and other mammals exposed to the product.

On May 11, 2001, an exemption from the requirement for a tolerance was established by EPA for Cry3Bb1 protein and the genetic material necessary for its production in corn (EPA, 2001). As part of the decision to grant this tolerance exemption EPA stated: "The lack of mammalian toxicity at high levels of exposure to the Cry3Bb1.....proteins demonstrate the safety of the product at levels well above maximum exposure levels anticipated in the crop". The Agency further stated: "There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry3Bb1.....proteins and the genetic material necessary for their production. This includes all anticipated dietary exposures and other exposures for which there is reliable information."

NPTII protein

Safety issues associated with use of *nptII* and the protein it expresses have previously been examined by ANZFA and the U.S. FDA in their ruling that authorised use of this gene product as a processing aid food additive for the development of transgenic tomatos, cotton and oilseed rape (FDA, 1994). This ruling was reviewed by a panel of scientific experts who concluded that the approach taken by FDA in evaluating the safety of *nptII* and the protein it expresses was scientifically sound and included all relevant parameters (FDA, 1998). The safety of NPTII has been addressed in multiple publications (Fuchs *et al.*, 1993a & 1993b; Flavel *et al.*, 1992; and Nap *et al.*, 1992). An acute oral NOEL ≥5,000 mg/kg has been established for NPTII protein in mice. The computed MOS for potential NPTII human exposure through consumption of MON 863 grain exceeds $8x10^7$.

The *nptII* gene is present in a number of commercial transgenic crops developed by Monsanto that have successfully completed the consultation process with FDA. Lastly, the EPA has established an exemption from the requirement of a tolerance for NPTII and the genetic material necessary for its expression in or on raw agricultural commodities (EPA, 1994). Collectively, these regulatory actions confirm the safety of the NPTII protein.

Study reports being submitted by Monsanto in support of this application include:

Bonnette, K. L. and P. D. Pyla (2001). An acute oral toxicity study in mice with *E. coli* produced Cry3Bb1.11098(Q349R) protein. MSL-17382, an unpublished study conducted for Monsanto Company.

Hileman, R. E., G. Holleschak, L. A. Turner, R. S. Thoma, C. R. Brown and J. D. Astwood (2001a). Characterization and equivalence of the Cry3Bb1 protein produced by *E. coli* fermentation and corn event MON 863. MSL-17274, an unpublished study conducted by Monsanto Company.

Thoma, R. S., G. Holleschak, R. E. Hileman and J. D. Astwood (2001). Primary structural protein characterization of corn event MON 863 Cry3Bb1.11098 protein using

N-terminal sequencing and MALDI time of flight mass spectrometric techniques. MSL-17154, an unpublished study conducted by Monsanto Company.

3.5.6 Amino acid sequence of novel proteins

Cry3Bb1 Protein

Nucleotides comprising the transgenic insert in event MON 863 have been sequenced by Monsanto (Cavato and Lirette, 2001). The amino acid sequence of the Cry3Bb1 variant produced in corn event MON 863 has been deduced from the nucleotide sequence and confirmed by N-terminal sequencing and MALDI-TOF (Thoma *et al.*, 2001). This variant shares 98.9% sequence identity with the wild type protein, differing by only seven of 653 amino acids (Hileman and Astwood, 2001).

Table 7 displays the complete amino acid sequences of Cry3Bb1 wild type protein and the Cry3Bb1 variant produced in corn containing event MON 863. To facilitate linkage of the redesigned *cry3Bb1* gene to a plant-effective promoter, it was necessary to create a restriction site at the 5' end of the coding sequence. Insertion of the nucleotides 'GCC' at positions 4, 5 and 6 in the coding sequence provides a *Nco* I restriction endonuclease cloning site. Insertion of this restriction site has the effect of introducing an alanine residue at position two of the amino acid terminal end of the protein. Thus, for the plant-produced variant of Cry3Bb1, numbering of amino acids appearing after alanine is shifted upward by one number when sequence aligning to similar proteins produced in *B.t.* Table 8 provides a key to the amino acid designations used in Table 7.

Table 7. This table compares amino acid sequences of Cry3Bb1 wild type protein and the Cry3Bb1 variant produced in corn event MON 863. Amino acid differences from the wild type protein are underscored (_) and shaded.

Protein	AA #	Amino Acid Code				
Wild type	1	M NPNNRSEH	DTIKVTPNSE	LQTNHNQYPL	ADNPNSTLEE	LNYKEFLRMT
MON 863	1	M_NPNNRSEH	DTIKVTPNSE	LQTNHNQYPL	ADNPNSTLEE	LNYKEFLRMT
Wild type	51	EDSSTEVLDN	STVKDAVGTG	ISVVGQILGV	VGVPFAGALT	SFYQSFLNTI
MON 863	51	EDSSTEVLDN	STVKDAVGTG	ISVVGQILGV	VGVPFAGALT	SFYQSFLNTI
Wild type	101	WPSDADPWKA	FMAQVEVLID	KKIEEYAKSK	ALAELQGLQN	NFEDYVNALN
MON 863	101	WPSDADPWKA	FMAQVEVLID	KKIEEYAKSK	ALAELQGLQN	NFEDYVNALN
Wild type	151	SWKKTPLSLR	SKRSQDRIRE	LFSQAESHFR	NSMPSFAVSK	FEVLFLPTYA
MON 863	151	SWKKTPLSLR	SKRSQ_RIRE	LFSQAESHFR	NSMPSFAVSK	FEVLFLPTYA
Wild type	201	QAANTHLLLL	KDAQVFGEEW	GYSSEDVAEF	YHRQLKLTQQ	YTDHCVNWYN
MON 863	201	QAANTHLLLL	KDAQVFGEEW	GYSSEDVAEF	Y_RQLKLTQQ	YTDHCVNWYN
Wild type	251	VGLNGLRGST	YDAWVKFNRF	RREMTLTVLD	LIVLFPFYDI	RLYSKGVKTE
MON 863	251	VGLNGLRGST	YDAWVKFNRF	RREMTLTVLD	LIVLFPFYDI	RLYSKGVKTE

Table 7 (cont.). This table compares amino acid sequences of Cry3Bb1 wild type protein and the Cry3Bb1 variant produced in corn event MON 863. Amino acid differences from the wild type protein are underscored (_) and shaded.

Protein	AA #	Amino Acid Code				
Wild type	301	LTRDIFTDPI	FSLNTLQEYG	PTFLSIENSI	RKPHLFDYLQ	GIEFHTRLQP
MON 863	301	LTRDIFTDPI	F_L_TLQ_YG	PTFLSIENSI	RKPHLFDYLQ	GIEFHTRL_P
Wild type	351	GYFGKDSFNY	WSGNYVETRP	SIGSSKTITS	PFYGDKSTEP	VQKLSFDGQK
MON 863	351	GYFGKDSFNY	WSGNYVETRP	SIGSSKTITS	PFYGDKSTEP	VQKLSFDGQK
Wild type	401	VYRTIANTDV	AAWPNGKVYL	GVTKVDFSQY	DDQKNETSTQ	TYDSKRNNGH
MON 863	401	VYRTIANTDV	AAWPNGKVYL	GVTKVDFSQY	DDQKNETSTQ	TYDSKRNNGH
Wild type	451	VSAQDSIDQL	PPETTDEPLE	KAYSHQLNYA	ECFLMQDRRG	TIPFFTWTHR
MON 863	451	VSAQDSIDQL	PPETTDEPLE	KAYSHQLNYA	ECFLMQDRRG	TIPFFTWTHR
Wild type	501	SVDFFNTIDA	EKITQLPVVK	AYALSSGASI	IEGPGFTGGN	LLFLKESSNS
MON 863	501	SVDFFNTIDA	EKITQLPVVK	AYALSSGASI	IEGPGFTGGN	LLFLKESSNS
Wild type	551	IAKFKVTLNS	AALLQRYRVR	IRYASTTNLR	LFVQNSNNDF	LVIYINKTMN
MON 863	551	IAKFKVTLNS	AALLQRYRVR	IRYASTTNLR	LFVQNSNNDF	LVIYINKTMN
Wild type	601	KDDDLTYQTF	DLATTNSNMG	FSGDKNELII	GAESFVSNEK	IYIDKIEFIP
MON 863	601	KDDDLTYQTF	DLATTNSNMG	FSGDKNELII	GAESFVSNEK	IYIDKIEFIP
Wild type	651	VQL				
MON 863	651	VQL				

Table 8. Key to amino acid designations used in Table 7.

A - Alanine	I - Isoleucine	R - Arginine
C - Cysteine	K - Lysine	S - Serine
D - Aspartic Acid	L - Leucine	T - Threonine
E - Glutamic Acid	M - Methionine	V - Valine
F - Phenylalanine	N - Asparagine	W - Tryptophan
G - Glycine	P - Proline	Y - Tyrosine
H - Histidine	Q - Glutamine	

NPTII protein

No new information is being provided.

3.5.7 Allergenic potential of novel proteins

B.t. Cry Proteins

Proteins of many sizes and function comprise a significant portion of the human diet. Only rarely do any of these tens of thousands of proteins elicit an allergic response when ingested (Taylor, 1992). Although there are currently no predictive bioassays available with which to assess the allergenic potential of proteins consumed by humans, a comparison of Cry3Bb1 structure to that of known allergens, toxins and pharmacologically active proteins can provide a basis for predicting the likelihood that ingestion of corn event MON 863 will elicit an adverse response in humans. Protein sequence databases were assembled for this purpose and included allergen and gliadin (ALLERGEN3), toxin (TOXIN4) and the public domain (ALLPEPTIDES) sequence databases.

The FASTA sequence alignment tool was used to assess structural similarity. Although the FASTA program directly compares amino acid sequences (*i.e.* primary protein structure), the alignment data may be used to infer higher order structure (*i.e.* secondary and tertiary protein structure). Proteins that share a high degree of similarity throughout the entire length are often homologous. Homologous proteins share secondary structure and common three-dimensional folds. Therefore, proteins homologous to allergens are more likely to share cross-reactive allergenic epitopes than are unrelated proteins.

Structural similarities between the Cry3Bb1 protein sequence and the aligned database sequences were examined (Hileman *et al.*, 2001b). The extent of each similarity was evaluated by visual inspection of the alignment, the calculated percent identity, and *E* score value. The Cry3Bb1 protein sequence was also screened against the ALLERGEN3 sequence database for immunologically relevant similarity using a pair-wise comparison algorithm. In these analyses, sequences of eight (or more) linearly contiguous and identical amino acids were defined as immunologically relevant (Metcalfe *et al.*, 1996; Hileman *et al.*, 2002). The presence of such identities may point to the presence of potentially cross-reactive allergenic epitopes.

No biologically relevant structural similarities were observed between any known allergen or toxin and the Cry3Bb1 protein produced in corn event MON 863. Further, no immunologically relevant sequence similarities were observed between this Cry3Bb1 protein and proteins in the allergen and gliadin database. These data demonstrate the absence of both structurally and immunologically relevant similarities between allergens and the Cry3Bb1 protein produced in corn event MON 863.

Apart from expected similarities to other known crystal proteins found in *B.t.* and related species, no additional significant structural similarities were observed. The Cry family represents a diverse set of proteins derived from a common ancestral gene. The results of these bioinformatics analyses indicate that the Cry3Bb1 protein produced by corn event MON 863 is not similar to known allergens, toxins or other pharmacologically active proteins relevant to animal or human health.

Current scientific knowledge suggests that food allergens are abundant, resistant to pepsin digestion, may be resistant to acid or heat, and can be glycosylated (Metcalfe *et al.*, 1996; Astwood *et al.*, 1996). The results of an *in vitro* digestive fate study indicate that Cry3Bb1 protein degrades to nondetectable levels in simulated gastric fluid within 15 seconds (Leach *et al.*, 2001). In simulated intestinal fluid, Cry3Bb1 is observed to degrade within one minute from a size of approximately 74 kDa to smaller fragments with approximate molecular

weights of 68 and 57 kDa. Continued exposure to simulated intestinal fluid resulted in the formation of a single stable fragment with an approximate molecular weight of 57 kDa which is the expected size of the trypitic core for Cry3 proteins (Hileman *et al.*, 2001c). Neither the Cry3Bb1 protein produced by *E. coli* nor MON 863 corn is glycosylated (Hileman *et al.*, 2001a). Cry3Bb1 is not detectable in MON 863 grain following baking at an elevated temperature for 30 minutes (Holleschak *et al.*, 2001).

Collectively, these data demonstrate that ingestion of Cry3Bb1-containing corn is unlikely to produce an allergic or toxic response in humans. There are also no confirmed cases of allergic reactions to Cry proteins in applicators of microbial-derived *B.t.* products during 40 years of use (McClintock et al., 1995).

NPTII protein

No new information is being submitted.

Conclusion on Safety of Novel Proteins

In summary, the human safety of Cry3Bb1 protein is demonstrated by: 1) the results of studies that show an absence of adverse effects in a mammalian specie following acute oral exposure, a very large margin of safety for projected dietary consumption, rapid digestion, and the absence of sequence similarities to known toxins and allergens; 2) comparable results to studies of other proteins in the Cry3 class, including Cry3Bb1 in the commercial product, *Raven Oil Flowable Bioinsecticide*; and 3) a nearly 50-year history of safe use for *B.t.* Cry proteins in U.S. agriculture. The weight of evidence establishes a reasonable certainty of no harm for this protein.

Both EPA and the U.S. Food and Drug Administration have previously determined that NPTII is safe for human and animal consumption (EPA, 1994; FDA, 1998).

Study reports being submitted by Monsanto in support of this application include:

Cavato, T.A. and Lirette, R.P. (2001) PCR analysis and DNA sequence of the insert in corn rootworm event MON 863. MSL-17108, an unpublished study conducted by Monsanto Company.

Hileman, R. E. and Astwood (2001). Additional Characterization of the Cry3Bb1 Protein Produced in Corn Event MON 863; Report MSL-17137, an unpublished study conducted by Monsanto Company.

Hileman, R. E., E. A. Rice, R. E. Goodman and J. D. Astwood (2001b). Bioinformatics Evaluation of the Cry3Bb1 Protein Produced in Corn Event MON 863 Utilizing Allergen, Toxin and Public Domain Protein Databases. Report MSL-17140, an unpublished analysis conducted by Monsanto Company.

Hileman, R. E., J. N. Leach and J. D. Astwood (2001c). Assessment of the *in vitro* digestibility of the Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid. MSL-17530, an unpublished study conducted by Monsanto Company.

Holleschak, G., R. Hileman and J. D. Astwood (2001). Amended report for MSL-16597: Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 proteins in the grain of insect protected corn events MON 863 and MON 853 after heat treatment. MSL-17223, an unpublished study conducted by Monsanto Company.

Leach, J. N., Hileman, R. E. and Astwood J. D. (2001). Assessment of the *in vitro* digestibility of Cry3Bb1 protein purified from corn event MON 863 and Cry3Bb1 protein purfied from *E. coli.* MSL-17292; an unpublished study conducted by Monsanto Company.

3.6 OTHER NOVEL SUBSTANCES

Refer to section 3.3.3 on nonexpressed genes for discussion on the presence of a portion of the bleomycin binding protein gene known as *ble*.

No other novel proteins or metabolites have been identified in the Corn Rootworm Protected Corn Event MON 863.

3.7 COMPARATIVE ANALYSIS

3.7.1 Compositional analysis

Compositional analyses were performed on tissues collected from corn event MON 863, its nontransgenic parental control line, and 18 commercial corn hybrids grown under field conditions (Ridley *et al.*, 2002). Field trials were conducted in the U.S. in 1999 at four replicated sites located in Monmouth, Illinois (MN); Richland, Iowa (RD); Van Horne, Iowa (VH); and York, Nebraska (YK). Corn event MON 863 and its parental control line were planted at all sites. Four different commercial reference hybrids (nontransgenic) were planted at two sites and five different commercial reference hybrids (nontransgenic) were planted at the remaining two sites to give a total of 18 different reference hybrids.

Forage and grain were collected from all sites. Compositional analyses were conducted to measure proximates (protein, fat, ash, moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acids, fatty acids, vitamin E, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc), phytic acid and trypsin inhibitor content of grain; and to measure proximate, ADF and NDF content of forage. In addition, the content of carbohydrates in forage and grain was determined by calculation. In all, 51 different components (7 in forage and 44 in grain) were evaluated as part of the safety and nutritional assessment of corn event MON 863.

Statistical analysis of the compositional data were conducted using a randomized complete block model analysis of variance for five sets of comparisons: analyses of data from each of the four replicated trials and data from a combination of all four trials. As there were a total of 51 components evaluated, a total of 255 comparisons were made: 51 comparisons for each of the five statistical analyses. The test event, MON 863, was compared to the control line, MON 846, to determine statistically significant differences at $p<0.05$. In addition, the comparison of MON 863 to the 95% tolerance interval for the commercial reference varieties was conducted to determine if the range of values for MON 863 fell within the population of

commercial maize. Refer to unpublished report MSL-17669 (Ridley *et al.*, 2002) for a detailed description of the materials and methods used for this investigation.

The results of compositional analyses revealed that all of the 51 components measured in corn event MON 863 were within the range of observed values for commercial corn lines planted at the same U.S. sites in 1999. Futhermore, all 51 components were within published literature ranges (Jugenheimer, 1976; Watson, 1982; Watson, 1987), or historical ranges for nontransgenic maize varieties. Tables 14 through 18 provide a summary of grain compositional results for all four sites combined. Table 19 provides a summary of forage compositional results for all four sites combined.

There were no statistically significant differences in 224 of the 255 comparisons made between corn event MON 863 and the parental control line which included forage (fat, protein, ash, carbohydrate, ADF and NDF) and grain (ash, ADF, NDF, 12 of 18 amino acids, six of eight fatty acids, potassium, and trypsin inhibitor) components. Differences computed for the remaining 31 comparisons were found to be statistically significant. The site and parameter combinations that displayed a statistically significant difference between MON 863 and parental control means are displayed in Table 20. Five percent or approximately thirteen (0.05×255) of these significant differences were expected to be false positives based on chance alone. Differences that were observed at only one to three of the sites, and not consistently across all four sites, are unlikely to be of biological significance. These differences between MON 863 and the parental controls, expressed as a percent of the control values, ranged from 1.38%-15.52%.

The range of values for those compositional components associated with small statistical differences were found to all fall within the 95% tolerance interval for commercial varieties planted at the same U.S. sites in 1999. This demonstrates, with a confidence level of 95%, that the levels of key nutrients and other compositional components for MON 863 were within a tolerance interval specified to contain 99% of the nontransgenic commercial reference corn hybrid population. Therefore, these minor differences are unlikely to be biologically meaningful, and the grain and forage from MON 863 are considered compositionally equivalent to that of conventional corn grain and forage.

Conclusion on Host Plant Safety

Extensive analyses have been performed to compare the composition of grain and forage from corn event MON 863 to that of nontransgenic varieties. The results of these analyses demonstrate that grain and forage from corn event MON 863 are substantially equivalent to nontransgenic corn. Taken together with corn's history of safe use as a common food and feed source, a conclusion of no significant risk is reached for the host plant in the safety assessment of this new variety.

Study reports being submitted by Monsanto in support of this application include:

> Ridley, W. R., M. A. Nemeth, J. D. Astwood, M. L. Breeze and R. Sorbet (2002). Amended report for MSL-17199: Compositional analyses of forage and grain collected from corn rootworm protected maize event MON 863 grown in 1999 U.S. field trials. MSL-17669, an unpublished study conducted by Monsanto Company.

Key to Interpreting Data Presented in Tables 14 through 20:

- MON 863 and control mean values are for 16 replicate samples collected from 4 sites
- S.E. = standard error of the mean
- C.I. = confidence interval
- Comm. = commercial value; the range of sample values for commercial lines grown at the same field sites in these 1999 trials
- T.I. = tolerance interval, specified to contain 95% of the commercial line population, negative limits set to zero
- Lit. = literature ranges (Jugenheimer, 1976; Watson, 1982; Watson, 1987)
- Historical range for control lines analyzed in Monsanto trials conducted between 1993 and 1995 (Sanders and Patzer, 1995; Sanders *et al.*, 1996a,b; 1997a,b,c). NOTE: These historical control reference studies are already on file with ANZFA authorities and are not being resubmitted as part of this dossier.

Table 14. Combined site statistical comparison of amino acid levels in MON 863 and control grain.

Amino Acid (% of total)	MON 863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 863 minus Control) Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
Alanine	7.74 ± 0.032 (7.65 - 7.85)	7.79 ± 0.032 (7.46 - 7.98)	-0.045 ± 0.031 (-0.23 – 0.24)	0.247	-0.14, 0.055	7.30 - 8.06 (6.94, 8.46)	6.4-9.9	7.2-8.8
Arginine	4.43 ± 0.062 (4.21 - 4.68)	4.33 ± 0.062 (4.09 - 4.63)	0.10 ± 0.044 (-0.16 – 0.51)	0.030	-0.0099, 0.19	3.86 - 4.83 (3.38, 5.22)	2.9-5.9	3.5-5.0
Aspartic acid	6.51 ± 0.053 (6.38 - 6.72)	6.45 ± 0.053 (6.30 - 6.67)	0.061 ± 0.021 (-0.11 – 0.23)	0.064	-0.0070, 0.13	6.05 - 7.14 (5.54, 7.65)	5.8-7.2	6.3-7.5
Cystine	2.20 ± 0.027 (1.98 - 2.40)	2.09 ± 0.027 (1.99 - 2.29)	0.11 ± 0.029 (-0.15 – 0.39)	<0.001	0.054, 0.17	1.84 - 2.35 (1.59, 2.65)	1.2-1.6	1.8-2.7
Glutamic acid	19.39 ± 0.16 (18.99 – 19.91)	19.56 ± 0.16 (18.97 - 20.26)	-0.17 ± 0.090 (-0.76 – 0.24)	0.157	-0.46, 0.12	18.31 - 20.25 (17.55, 21.25)	12.4-19.6	18.6-22.8
Glycine	3.60 ± 0.048 (3.45 - 3.74)	3.53 ± 0.048 (3.32 - 3.72)	0.072 ± 0.030 (-0.075 - 0.31)	0.100	-0.025, 0.17	3.20 - 4.13 (2.81, 4.46)	2.6-4.7	3.2-4.2
Histidine	2.84 ± 0.032 (2.70 – 2.95)	2.83 ± 0.032 (2.72 - 2.94)	0.011 ± 0.023 (-0.082 - 0.24)	0.665	-0.063, 0.085	2.60 - 3.20 (2.37, 3.35)	2.0-2.8	2.8-3.4
Isoleucine	3.67 ± 0.033 (3.45 – 3.89)	3.74 ± 0.033 (3.61 - 3.87)	-0.064 ± 0.033 (-0.33 – 0.15)	0.072	-0.13, 0.0065	3.47 - 3.94 (3.20, 4.17)	2.6-4.0	3.2-4.3
Leucine	13.36 ± 0.081 (12.88 – 13.65)	13.65 ± 0.081 (13.27 - 14.17)	-0.29 ± 0.084 (-0.75 – 0.13)	0.039	-0.56, -0.026	11.94 - 14.47 (11.30, 15.63)	7.8-15.2	12.0-15.8

(continued next page)

Table 14 (cont.). Combined site statistical comparison of amino acid levels in MON 863 and control grain.

Amino Acid (% of total)	MON 863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 863 minus Control) Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Lit. Range	Historical Range
Lysine	2.92 ± 0.061 (2.65 - 3.26)	2.88 ± 0.061 (2.67 - 3.08)	0.042 ± 0.036 (-0.19 – 0.32)	0.328	-0.073, 0.16	2.40 - 3.52 (1.87, 3.89)	2.0-3.8	2.6-3.5
Methionine	2.28 ± 0.060 (1.89 - 2.49)	2.24 ± 0.060 (1.96 - 2.58)	0.034 ± 0.035 (-0.20 – 0.25)	0.348	-0.040, 0.11	1.61 - 2.29 (1.34, 2.74)	1.0-2.1	1.3-2.6
Phenylalanine	4.99 ± 0.015 (4.93 - 5.06)	5.04 ± 0.015 (4.95 - 5.23)	-0.048 ± 0.017 (-0.17 – 0.041)	0.052	-0.096, 0.0010	4.80 - 5.35 (4.53, 5.66)	2.9-5.7	4.9-6.1
Proline	8.73 ± 0.054 (8.30 - 9.21)	8.78 ± 0.054 (8.60 - 9.05)	-0.052 ± 0.046 (-0.32 – 0.38)	0.267	-0.15, 0.045	8.57 - 9.61 (8.04, 10.35)	6.6-10.3	8.7-10.1
Serine	4.70 ± 0.11 (3.93 - 5.09)	4.67 ± 0.11 (4.20 - 4.94)	0.031 ± 0.094 (-0.77 - 0.89)	0.743	-0.17, 0.23	4.24 - 4.99 (3.76, 5.69)	4.2-5.5	4.9-6.0
Threonine	3.41 ± 0.035 (3.16 - 3.60)	3.36 ± 0.035 (3.16 - 3.49)	0.049 ± 0.024 (-0.15 - 0.23)	0.056	-0.0016, 0.099	3.19 - 3.59 (2.93, 3.83)	2.9-3.9	3.3-4.2
Tryptophan	0.66 ± 0.015 (0.60 - 0.83)	0.65 ± 0.015 (0.60 - 0.68)	0.013 ± 0.012 (-0.043 - 0.17)	0.295	-0.013, 0.039	0.54 - 0.82 (0.37, 0.90)	0.5-1.2	0.4-1.0
Tyrosine	3.63 ± 0.057 (3.33 - 3.77)	3.48 ± 0.057 (2.71 - 3.82)	0.15 ± 0.078 (-0.14 - 0.92)	0.073	-0.016, 0.32	2.60 - 3.73 (2.15, 4.65)	2.9-4.7	3.7-4.3
Valine	4.94 ± 0.043 (4.71 - 5.13)	4.94 ± 0.043 (4.64 - 5.12)	-0.0091 ± 0.043 (-0.36 - 0.50)	0.833	-0.097, 0.079	4.49 - 5.30 (4.15, 5.63)	2.1-5.2	4.2-5.3

Table 15. Combined site statistical comparison of fatty acid levels in MON 863 and control grain.

Fatty Acid (% of total)	MON 863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 863 minus Control) Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
16:0 palmitic acid	12.01 ± 0.11 (11.61 – 12.56)	11.88 ± 0.11 (11.66 - 12.20)	0.12 ± 0.11 (-0.21 - 0.79)	0.337	-0.22, 0.47	9.07 - 12.14 (7.74, 13.87)	7-19	9.9-12.0
18:0 stearic acid	1.66 ± 0.083 (1.40 - 1.86)	1.66 ± 0.083 (1.33 - 1.81)	0.0044 ± 0.013 (-0.087 - 0.078)	0.738	-0.023, 0.032	1.44 - 2.40 (1.04, 2.68)	1-3	1.4-2.2
18:1 oleic acid	22.00 ± 0.36 (20.97 – 23.55)	21.87 ± 0.36 (21.00 - 22.53)	0.13 ± 0.12 (-0.16 - 1.05)	0.365	-0.26, 0.52	21.26 - 32.06 (13.28, 36.31)	20-46	20.6-27.5
18:2 linoleic acid	62.23 ± 0.38 (60.02 – 63.21)	62.47 ± 0.38 (61.55 - 63.60)	-0.23 ± 0.18 (-1.83 - 0.32)	0.293	-0.81, 0.35	54.15 - 63.64 (50.21, 70.86)	35-70	55.9-66.1
18:3 linolenic acid	1.20 ± 0.020 (1.13 - 1.29)	1.24 ± 0.020 (1.09 - 1.45)	-0.037 ± 0.021 (-0.30 - 0.071)	0.079	-0.080, 0.0047	0.97 - 1.36 (0.75, 1.51)	0.8-2	0.8-1.1
20:0 arachidic acid	0.41 ± 0.0068 (0.39 - 0.44)	0.40 ± 0.0068 (0.39 - 0.42)	0.0052 ± 0.0062 (-0.017 - 0.027)	0.460	-0.014, 0.025	0.35 - 0.45 (0.30, 0.51)	0.1-2	0.3-0.5
20:1 eicosenoic acid	0.30 ± 0.011 (0.28 - 0.35)	0.30 ± 0.011 (0.28 - 0.35)	0.0011 ± 0.0037 (-0.039 - 0.040)	0.783	-0.011, 0.013	0.25 - 0.39 (0.18. 0.42)	na	0.2-0.3
22:0 behenic acid	0.18 ± 0.0068 (0.17 - 0.21)	0.18 ± 0.0068 (0.15 - 0.21)	0.0043 ± 0.0056 (-0.023 - 0.029)	0.498	-0.013, 0.222	0.089 - 0.21 (0.055, 0.30)	na	0.1-0.3

Table 16. Combined site statistical comparison of mineral levels in MON 863 and control grain.

Mineral	MON 863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 863 minus Control) Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
Calcium (% dw)	0.0052 ± 0.00041 (0.0041 – 0.0064)	0.0053 ± 0.00041 (0.0043 - 0.0089)	-0.00013 ± 0.00020 (-0.0027 - 0.00081)	0.538	-0.00056, 0.00031	0.0039 - 0.0060 (0.0022, 0.0073)	0.01-0.1	0.003-0.006
Copper (mg/kg dw)	2.26 ± 0.17 (1.72 - 3.18)	2.19 ± 0.17 (1.60 - 2.88)	0.078 ± 0.076 (-0.58 - 1.10)	0.315	-0.078, 0.23	1.03 - 2.15 (0.25, 2.70)	0.9-10	na
Iron (mg/kg dw)	23.55 ± 1.16 (21.13 – 26.36)	24.18 ± 1.16 (20.57 - 28.16)	-0.63 ± 0.80 (-3.92 - 1.83)	0.490	-3.18, 1.92	16.74 - 28.69 (12.52, 35.06)	1-100	na
Magnesium (% dw)	0.13 ± 0.0034 (0.12 - 0.14)	0.14 ± 0.0034 (0.12 - 0.16)	-0.0049 ± 0.0024 (-0.018 - 0.0049)	0.135	-0.013, 0.0028	0.091 - 0.14 (0.082, 0.17)	0.09-1.0	na
Manganese (mg/kg dw)	5.81 ± 0.78 (3.75 - 7.40)	6.15 ± 0.78 (4.01 - 8.28)	-0.34 ± 0.16 (-0.94 - 0.58)	0.122	-0.84, 0.17	3.51 - 9.80 (0, 12.84)	0.7-54	na
Phosphorus (% dw)	0.40 ± 0.0068 (0.37 - 0.45)	0.42 ± 0.0068 (0.39 - 0.46)	-0.022 ± 0.0094 (-0.070 - 0.019)	0.065	-0.045, 0.0020	0.27 - 0.41 (0.21, 0.47)	0.26-0.75	0.288-0.363
Potassium (% dw)	0.43 ± 0.0088 (0.40 - 0.48)	0.44 ± 0.0088 (0.39 - 0.48)	-0.0074 ± 0.0087 (-0.056 - 0.037)	0.457	-0.035, 0.020	0.33 - 0.43 (0.28, 0.48)	0.32-0.72	na
Zinc (mg/kg dw)	22.15 ± 1.44 (17.95 – 25.25)	23.68 ± 1.44 (18.77 – 28.14)	-1.53 ± 0.69 (-4.60 - 0.90)	0.112	-3.73, 0.66	12.84 - 31.22 (6.31, 37.95)	12-30	na

Table 17. Combined site statistical comparison of fiber and proximate content in MON 863 and control grain.

Fiber & Proximates	MON 863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 863 minus Control) Mean ± S.E. (Range)	p-value	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
Ash (% dw)	1.35 ± 0.12 (0.84 - 1.71)	1.41 ± 0.12 (0.89 - 1.89)	-0.064 ± 0.047 (-0.45 – 0.31)	0.196	-0.17, 0.037	0.62 - 1.53 (0.26, 2.06)	1.1-3.9	1.2-1.8
Carbohydrates (% dw)	83.30 ± 0.56 (81.83 – 85.00)	82.76 ± 0.56 (80.70 - 84.80)	0.54 ± 0.27 (-0.78 - 2.43)	0.138	-0.32, 1.40	82.51 - 87.84 (78.97, 90.36)	na	81.7-86.3
Acid detergent fiber (% dw)	4.45 ± 0.15 (3.49 - 5.23)	4.50 ± 0.15 (3.62 - 5.89)	-0.050 ± 0.18 (-1.77 - 1.16)	0.778	-0.43, 0.33	3.65 - 6.09 (1.98, 6.62)	3.3 - 4.3	3.1 - 5.3
Neutral detergent fiber (% dw)	11.64 ± 0.54 (9.21 – 13.47)	12.02 ± 0.54 (10.31 - 15.82)	-0.37 ± 0.61 (-4.32 - 2.30)	0.585	-2.33, 1.58	9.50 - 14.95 (6.51, 16.28)	8.3-11.9	9.6 - 15.3
Moisture (% fw)	10.03 ± 0.50 (8.54 – 11.20)	10.23 ± 0.50 (8.60 - 11.40)	-0.20 ± 0.13 (-0.90 - 0.26)	0.216	-0.61, 0.21	8.75 - 15.70 (5.09, 18.62)	7-23	9.4 - 15.8
Total fat (% dw)	3.77 ± 0.20 (3.00 - 4.56)	3.64 ± 0.20 (3.05 - 4.29)	0.13 ± 0.18 (-0.77 - 1.02)	0.520	-0.44, 0.70	2.18 - 3.86 (1.68, 4.64)	3.1-5.7, 2.9-6.1	2.4-4.2
Protein (% dw)	11.60 ± 0.48 (10.43 – 12.82)	12.19 ± 0.48 (10.45 - 13.80)	-0.59 ± 0.22 (-1.52 - 0.12)	0.071	-1.28, 0.097	- 13.83 (5.47, 16.57)	6.0 - 12.0, 9.7 – 16.1	9.0 - 13.6

Table 18. Combined site statistical comparison of nutrient and anti-nutrient factor levels in MON 863 and control grain.

Component	MON 863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 863 minus Control) Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
Phytic Acid (% dw)	1.11 ± 0.033 (0.92 - 1.28)	1.23 ± 0.033 (1.01 - 1.37)	-0.12 ± 0.034 (-0.31 - 0.19)	0.001	-0.19, -0.050	0.73 - 1.17 (0.39, 1.33)	to 0.9%	na
Trypsin Inhibitor (TIU/mg dw)	2.30 ± 0.16 (0.56 - 3.10)	2.48 ± 0.16 (1.91 - 3.45)	-0.18 ± 0.16 (-1.70 - 0.63)	0.288	-0.53, 0.17	0.58 - 3.05 (0, 4.25)	na	na
Vitamin E (mg/gdw)	0.011 ± 0.0012 (0.0062 – 0.014)	0.013 ± 0.0012 (0.0088 - 0.016)	-0.0015 ± 0.00047 (-0.0077 - 0.00090)	0.002	-0.0025, -0.00058	0.0041 - 0.014 (0, 0.019)	0.017-0.047	0.008-0.015

Table 19. Combined site statistical comparison of fiber and proximate content in MON 863 and control forage.

Component	MON 863 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 863 minus Control) Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Historical Range
Ash (% dw)	4.73 ± 0.22 (3.62 - 5.65)	5.00 ± 0.22 (3.81 - 6.27)	-0.27 ± 0.16 (-1.29 - 1.09)	0.106	-0.61, 0.066	3.74 - 5.02 (3.04, 5.58)	2.9 - 5.1
Carbohydrates (% dw)	84.24 ± 0.53 (82.29 - 86.32)	84.32 ± 0.53 (80.78 - 87.21)	-0.084 ± 0.43 (-2.70 - 2.52)	0.859	-1.47, 1.30	82.59 - 87.10 (81.22, 88.97)	84.6 - 89.1
Acid detergent fiber (% dw)	28.67 ± 1.66 (21.74 - 43.30)	28.41 ± 1.66 (23.39 - 32.08)	0.26 ± 2.06 (-7.90 - 14.03)	0.907	-6.29, 6.81	19.78 - 39.00 (9.33, 45.44)	21.4 - 29.2
Neutral detergent fiber (% dw)	43.25 ± 1.26 (37.97 - 49.67)	42.94 ± 1.26 (37.32 - 51.85)	0.31 ± 1.25 (-10.81 - 12.34)	0.807	-2.25, 2.87	30.30 - 47.75 (22.71, 56.02)	39.9 - 46.6
Moisture (% fw)	71.09 ± 0.46 (69.30 - 73.10)	71.68 ± 0.46 (69.80 - 74.50)	-0.58 ± 0.43 (-3.70 - 2.90)	0.269	-1.95, 0.79	67.00 - 74.10 (62.70, 77.69)	68.7 - 73.5
Protein (% dw)	8.62 ± 0.53 (6.91 - 10.40)	8.33 ± 0.53 (5.99 - 10.55)	0.30 ± 0.37 (-2.54 - 2.42)	0.478	-0.87, 1.47	6.45 - 10.14 (4.94, 11.97)	4.8 - 8.4
Total fat (% dw)	2.40 ± 0.23 (0.92 - 3.16)	2.35 ± 0.23 (1.30 - 3.33)	0.053 ± 0.15 (-0.91 - 1.14)	0.721	-0.26, 0.36	1.39 - 2.62 (1.03, 3.24)	1.4 - 2.1

Table 20. Summary of statistically significant differences in composition between MON 863 and parental control values.

Tissue/ Component	Site Code	MON 863 Mean	Parental Control Mean	Mean Difference (MON 863 minus Control)	Significance (*p-value*)	Mean Difference (% of Control Value)	MON 863 (Range)	Commercial Range (95% T.I. Lower, Upper)
Forage								
Moisture (% fw)	RD	70.23	71.43	-1.20	0.023	-1.68	(69.80 - 70.50)	(62.70, 77.69)
Grain								
Cystine (% Total AA)	MN	2.18	2.03	0.15	0.012	7.39	(2.15 - 2.21)	(1.59, 2.65)
Leucine (% Total AA)	MN	13.17	13.59	-0.42	0.013	-3.09	(12.88 - 13.42)	(11.30, 15.63)
Phenylalanine (% Total AA)	MN	4.99	5.09	-0.093	0.038	-1.83	(4.93 - 5.06)	(4.53, 5.66)
Zinc (mg/kg dw)	MN	20.51	22.79	-2.28	0.038	-10.00	(19.71 - 21.41)	(6.31, 37.95)
Total Fat (% dw)	MN	3.87	3.35	0.52	0.046	15.52	(3.59 - 4.06)	(1.68, 4.64)
Phytic Acid (% dw)	MN	1.15	1.33	-0.18	0.027	-13.53	(1.08 - 1.21)	(0.39, 1.33)
Leucine (% Total AA)	RD	13.44	13.67	-0.23	0.023	-1.68	(13.33 - 13.63)	(11.30, 15.63)
Protein (% dw)	RD	11.82	12.16	-0.34	0.039	-2.80	(11.63 - 11.97)	(5.47, 16.57)
Vitamin E (mg/g dw)	RD	0.013	0.015	-0.0023	0.011	-15.33	(0.012 - 0.014)	(0, 0.019)
18:3 linolenic (% Total FA)	RD	1.26	1.28	-0.020	0.043	-1.56	(1.23 - 1.29)	(0.75, 1.51)
Cystine (% Total AA)	VH	2.25	2.15	0.11	0.001	5.12	(2.22 - 2.29)	(1.59, 2.65)
20:0 Arachidic (% Total FA)	VH	0.43	0.41	0.022	0.001	5.37	(0.43 - 0.44)	(0.30, 0.51)
Iron (mg/kg dw)	VH	21.73	21.20	0.53	0.013	2.50	(21.13 - 23.05)	(12.52, 35.06)
Total Fat (% dw)	VH	3.08	3.42	-0.34	0.037	-9.94	(3.00 - 3.24)	(1.68, 4.64)
Moisture (% fw)	VH	9.86	10.37	-0.51	0.039	-4.92	(9.38 - 10.30)	(5.09, 18.62)
Aspartic Acid (% Total AA)	YK	6.44	6.36	0.088	0.040	1.38	(6.42 - 6.47)	(5.54, 7.65)
Tyrosine (% Total AA)	YK	3.67	3.48	0.19	0.026	5.46	(3.59 - 3.74)	(2.15, 4.65)
Calcium (% dw)	YK	0.0044	0.0047	-0.00023	0.035	-4.89	(0.0041 - 0.0047)	(0.0022, 0.0073)
Copper (mg/kg dw)	YK	1.85	1.69	0.16	0.002	9.47	(1.72 - 2.01)	(0.25, 2.70)

(continued next page)

Table 20 (cont.). Summary of statistically significant differences in composition between MON 863 and parental control values.

Tissue/ Component	Site Code	MON 863 Mean	Parental Control Mean	Mean Difference (MON 863 minus Control)	Significance (*p-value*)	Mean Difference (% of Control Value)	MON 863 (Range)	Commercial Range (95% T.I. Lower, Upper)
Grain (cont.)								
Iron (mg/kg dw)	YK	24.87	27.45	-2.58	0.013	-9.40	(23.99 - 25.42)	(12.52, 35.06)
Manganese (mg/kg dw)	YK	7.17	7.91	-0.75	0.012	-9.48	(6.94 - 7.40)	(0, 12.84)
Phosphorus (% dw)	YK	0.39	0.43	-0.036	0.037	-8.37	(0.37 - 0.41)	(0.21, 0.47)
Zinc (mg/kg dw)	YK	24.20	27.16	-2.96	0.013	-10.90	(23.54 - 25.25)	(6.31, 37.95)
Carbohydrate (% dw)	YK	82.56	81.28	1.28	0.046	1.57	(81.83 - 83.13)	(78.97, 90.36)
Protein (% dw)	YK	12.44	13.62	-1.18	0.009	-8.66	(12.19 - 12.82)	(5.47, 16.57)
Arginine (% Total AA)	All	4.43	4.33	0.10	0.030	2.31	(4.21 – 4.68)	(3.38, 5.22)
Cystine (% Total AA)	All	2.20	2.09	0.11	<0.001	5.26	(1.98 - 2.40)	(1.59, 2.65)
Leucine (% Total AA)	All	13.36	13.65	-0.29	0.039	-2.12	(12.88 - 13.65)	(11.30, 15.63)
Phytic Acid (% dw)	All	1.11	1.23	-0.12	0.001	-9.76	(0.92 - 1.28)	(0.39, 1.33)
Vitamin E (mg/g dw)	All	0.011	0.013	-0.0015	0.002	-11.54	(0.0062 - 0.014)	(0, 0.019)

3.7.2 Other constituents

No additional or novel constituents have been identified in corn rootworm protected corn event MON 863.

3.7.3 Allergenic protein levels

There have been a limited number of reports that corn contains naturally occurring allergenic proteins. Refer to section 3.5.7 for discussion on the allergenic potential of novel proteins.

3.8 NUTRITIONAL IMPACT

There is no significant change in the nutritional aspects of the corn rootworm protected corn event MON 863. There was no biologically significant change identified in nutrients or anti-nutrients and no change is expected in the consumption of corn or corn food products as part of normal dietary intake as a result of this new variety entering the food chain. Two animal feeding studies are presented which confirm that corn grain derived from the CRW protected corn is nutritionally equivalent to conventional corn.

An eight-week study was conducted to assess the growth and survival of channel catfish fed a diet containing CRW-protected corn grain (Li and Robinson, 1999). The test grain contained either of two transformation events, MON 853 or MON 859. Event MON 859 encoded the same Cry3Bb1 variant as produced in MON 863 corn. Event MON 853 encoded a Cry3bb1 variant that differed in its amino acid sequence from the MON 863 variant by only two amino acids. Nontransgenic corn grain of comparable genetics served as the control substances. Grain from a nontransgenic commercial hybrid, H2349, was used as a reference substance. No significant differences in feed consumption, weight gain, feed conversion ratio, survival, percent visceral fat, or overall percent fat, protein, and ash were observed among channel catfish fed test and control diets.

Significant differences were observed in percentage fillet moisture among fish fed different diets. Fish fed the diet containing MON 859 corn grain had a lower fillet moisture than fish fed the diet of control corn grain. Fillet moisture of fish fed MON 853 diet was not different from those fed control diet. In addition, fish fed MON 853 or MON 859 grain diets had a similar percentage fillet moisture content to fish fed the reference substance, H2349. The apparent statistical differences in fillet moisture are minimal and unlikely to be diet related. These data indicate that corn grain derived from the two CRW-protected corn events, MON 853 and MON 859, can be used as a feed ingredient in channel catfish diet at levels of up to 35% without adverse effect on fish growth, feed conversion efficiency, survival, behavior, or body composition.

The wholesomeness of corn grain containing event MON 863 was compared to six nontransgenic commercial corn varieties in a Ross x Ross broiler chicken feeding study (Taylor *et al.*, 2001b). The composition of grain used on this study was characterized by Taylor *et al.* (2001a). The results of this feeding study showed that all performance parameters measured were similar ($p>0.05$) across the broilers fed diets of MON 863 corn, parental control corn, and corn from commercially available reference varieties. In addition, broilers fed diets containing MON 863 corn had similar feed efficiency to the parental (LH82 x A634) and all reference lines, and similar adjusted feed efficiency to the parental and two

of the six commercially available reference lines (RX826 and DK539). The other four reference corn diets had slightly increased adjusted feed efficiencies (on average, 1.9% greater than MON 863). However, all feed efficiency and adjusted feed efficiency values fell within historical ranges for previous broiler studies, and literature ranges reported for feed efficiency.

Comparison of the MON 863 corn fed population to reference diets showed no differences in all performance and meat quality parameters and in most carcass yield parameters measured. There was a significant difference ($p<0.05$) in the fat pads of females (on a weight basis and percent of live weight basis) between MON 863 and the population of reference diets. These minor differences in the females were not observed in the males, and all mean values fell within the historical range of previous broiler studies and literature ranges reported for fat pad measurements. Therefore, this difference was not considered biologically significant as it falls within the natural variability of fad pad measurements of broiler chickens.

In conclusion, there were no biologically relevant differences in the parameters tested between broilers fed corn grain containing event MON 863 and conventional corn grain. In addition, when individual treatment comparisons were made, broilers in general had similar carcass yield and meat composition with diets containing MON 863, the parental control, or five commercially available reference lines. Corn event MON 863 was as wholesome as its corresponding nontransgenic parental line and six commercially available reference lines regarding ability to support the rapid growth of broiler chickens.

Study reports being submitted by Monsanto in support of this application:

Li, M. H. and E. H. Robinson (1999). Evaluation of insect protected corn lines MON 853 and MON 859 as a feed ingredient for catfish. MSL-16164, an unpublished study conducted by Monsanto Company.

Taylor, M. L., J. D. Astwood, M. Breeze and C. Stone (2001a). Pesticide profile, mycotoxin and compositional analysis of corn event MON 863 and control lines LH82xA634 produced in Kihei, Hawaii in 2000. MSL-16953, an unpublished study conducted by Monsanto Company.

Taylor, M. L., G. F. Hartnell, S. G. Riordan, M. A. Nemeth, T. A. Cavato, K. Karunanadaa, B. George, D. M. Carpenter and J. D. Astwood (2001b). Sponsor summary report for study #00-01-39-38: Comparison of broiler performance when fed diets containing event MON 863, nontransgenic parental line or commercial corn. MSL-17243, an unpublished study conducted by Monsanto Company.

3.9 DETECTION OF GENETIC MODIFICATION

An ELISA method can be used to estimate the levels of Cry3Bb1 and NPTII proteins in tissues of MON 863 and nontransgenic corn (Kolwyck *et al.*, 2001). This method can be used for estimating potential environmental and human exposures to the proteins.

In a study by Holleschak *et al.* (2001) the immunodetectability and bioactivity of the Cry3Bbl proteins in the grain of corn events MON 853 and MON 863 after heat treatment was investigated. Grain containing events MON 853 and MON 863 and the corresponding

conventional grain were ground to a fine powder and baked at 204 °C (399.2 °F) for 30 minutes to simulate a heat step used in food processing. Unbaked and baked grain of events MON 853 and MON 863 and control lines were extracted and analyzed by immunoblotting and ELISA to evaluate the immunodetectability of the Cry3Bbl proteins.

After heating MON 853 and MON 863 flour, Cry3Bbl protein was not detected using immunoblot analysis, and only 0.4% and 0% of the original Cry3Bbl protein content was detected by ELISA analysis. In contrast, the Cry3Bbl protein was readily detected using immunoblot and ELISA analysis for unbaked MON 853 and MON 863 flour. A 74 kDa band was detected using immunoblot analysis for unbaked MON 853 and MON 863 flour, suggesting that the full-length protein was extractable from grain. These results were consistent with the observed loss of insecticidal activity against Colorado potato beetles (*Leptinotarsa decemlibeatu*) in baked flour derived from MON 853 and MON 863 grain.

Study reports being submitted by Monsanto in support of this application:

Holleschak, G., R. E. Hileman and J. D. Astwood (2001). Amended report for MSL-16597: Immuno-detectability of Cry3Bb1.11098 and Cry3Bb1.11231 proteins in the grain of insect protected corn events MON 863 and MON 853 after heat treatment. MSL-17223, an unpublished study conducted by Monsanto Company.

Kolwyck, D., B-P. Tonnu, Y. A. Dudin, T. Ploesser and K. Gustafson (2001). Validated method for extraction and direct ELISA analysis of Cry3bb1 in corn grain.

3.10 MARKET IMPACT

The extent of human exposure to food products containing Cry3Bb1 protein in Australia and New Zealand is expected to be minor (see Section 3.2.3 above). Monsanto does not intend to commercialize corn hybrids for planting in Australia since the CRW pest does not inhabit this continent.

4.0 REGULATORY CONSIDERATIONS

4.1 OTHER APPROVALS

4.1.1 Overseas Regulatory Status

United States of America

Cry3Bb1 protein and corn event MON 863 have been undergoing regulatory review in the U.S. An exemption from the requirement of a tolerance (*i.e.*, maximum residue limit) was established by EPA for Cry3Bb1 residues in corn food and feed commodities based on a reasonable certainty that no harm will result from human aggregate exposure to this protein (EPA, 2001; 40 CFR §180.1214). The EPA has also granted an experimental use permit for field testing of MON 863 on up to 9,400 acres. A decision on registration of Cry3Bb1 protein and the genetic material necessary for its production in corn event MON 863 is pending at EPA.

On December 31, 2001, a food safety and nutritional assessment consultation for corn event MON 863 was completed with FDA. This consultation established that corn varieties containing event MON 863 are not materially different in composition, safety and other relevant parameters from corn currently on the market, and that MON 863 corn does not raise issues that would require premarket review or approval by FDA.[2]

On October 8, 2002, the USDA issued a determination of nonregulated status for corn varieties containing event MON 863.

Canada

Applications for commercialization of corn event MON 863 have been submitted to the Canadian Food Inspection Agency and Health Canada.

Japan

Approvals necessary for the importation of corn commodities and food or feed products containing event MON 863 have been granted by the Japanese Ministry of Agriculture, Forestry and Fisheries and the Ministry of Health, Labor and Welfare.

European Union

Applications for importation of corn commodities and food or feed products containing event MON 863 have been submitted to the competent authorities in the European Union.

Argentina and Chile

Permits have been granted for lanting of field research and seed production trials in Chile, and for field research trials in Argentina.

[2] FDA letter to Monsanto; http://www.cfsan.fda.gov/~rdb/bnfL075.html

4.1.2 Regulatory Rejection or Withdrawal

There have been no rejections or withdrawals of applications for registration of corn rootworm protected corn event MON 863.

4.2 REGULATORY IMPACT STATEMENT

4.2.1 Cost Implications

Corn (*i.e.* maize) is the world's third leading cereal crop, following wheat and rice. It is grown as a commercial crop in over 25 countries. Worldwide production of corn is approximately 587 million metric tons a year (Corn Refiners Association, 2002). The U.S. accounts for approximately 41% of global grain production. Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2001, corn production covered 75.8 million acres (30.6 million ha), yielding 9.5 billion bushels, at an average yield of 138.2 bu/ac, and total net value of US$19.0 billion (NCGA, 2002).

Corn yields are negatively impacted by a number of insect pests. One of the most pernicious in the U.S. Corn Belt is the CRW. CRW larvae damage corn by feeding on the roots, reducing the ability of the plant to absorb water and nutrients from soil, and causing harvesting difficulties due to plant lodging (Reidell, 1990; Spike and Tollefson, 1991). CRW is the most significant insect pest problem for corn production in the U.S. from the standpoint of chemical insecticide usage (Ward, 2002). Approximately 14 million acres of corn were treated with organophosphate, carbamate and pyrethroid insecticides to control CRW in 2000. CRW has been described as the billion dollar (US$) pest complex, based on costs associated with the application of soil insecticides and crop losses due to pest damage (Metcalf, 1986).

Corn growers mitigate CRW damage primarily through crop rotation or the use of soil insecticides. Historically, crop rotation has provided highly effective protection from CRW damage in many agronomic situations. However, several factors now limit the usefulness of this management strategy. First, many growers prefer the option of continuous (*i.e.*, nonrotated) corn production, even if this practice requires increased chemical inputs for soil fertility and insect control. Second, researchers have confirmed the existence of a northern corn rootworm variant that exhibits an extended diapause period. The eggs of this variant are able to survive through the noncorn years of crop rotation to yield beetles that affect first year corn (Ostlie, 1987; Tollefson, 1988; Gray *et al.*, 1998). Third, and of critical importance, crop rotation is no longer effective as a cultural corn rootworm management option in east central Illinois and northern Indiana due to the rapid spread of a new strain of western corn rootworm that, unlike previous populations, lays its eggs in soybean fields (Onstad and Joselyn, 1999; O'Neal *et al.*, 1999). The eggs of this western corn rootworm variant overwinter in soybean fields and emerge the following year in corn. Based on the rapid expansion of this variant population since its initial discovery in Illinois, it is expected to continue to spread throughout the Corn Belt.

Each of these factors has increased grower reliance on chemical insecticides for CRW control. The most common insecticide regime is the application of a granular soil insecticide, either in-furrow or banded, at the time of planting. In some agronomic situations, pesticide sprays are applied for adult suppression. The National Agricultural Statistics Service of the

USDA has compiled statistics on 2001 corn insecticide use across 19 states comprising 70.7 million acres of corn (NASS, 2002). These statistics indicate that chemical insecticides registered for CRW control were applied on over 32% of this corn acreage in 2001. Many of these insecticides have come under increased regulatory scrutiny because of concerns about worker safety, environmental risk, and exposure to infants and children.

The benefits offered by commercialization of this technology are substantial. Corn hybrids containing event MON 863 are consistently more efficacious than soil-applied insecticides in protecting roots from larval feeding damage (Ward, 2002). This superior performance is expected to result in a significant yield advantage for growers planting MON 863 hybrids. Preliminary estimates place this yield benefit at 1.5 to 4.5%. For a reasonable range of prices and yields, the value of this yield benefit to growers is US$4-12/ac relative to the use of a soil-applied insecticide, depending on CRW pressure (Mitchell, 2002).

Cry3Bb1 is far less hazardous than all insecticide active ingredients currently approved for corn rootworm control. The majority of corn rootworm insecticides are classified as Restricted Use and virtually all are undergoing reassessment by multiple regulatory authorities due to significant environmental risks. The adoption of MON 863 hybrids provides an opportunity to tremendously reduce the occupational and environmental risks currently associated with the manufacture, transportation, storage, handling, application and disposal of conventional insecticides. In excess of seven million pounds of insecticide active ingredient are annually applied to 14 million acres of corn for the control of corn rootworms in the U.S. At product maturity, MON 863 hybrids have the potential to reduce these applications by millions of pounds (Ward, 2002). Regulatory approval of plant-incorporated Cry3Bb1 to limit crop losses caused by corn rootworms will provide substantial benefits for growers and a reasonable certainty of no harm for humans and the environment.

4.2.2 Profit Implications

Corn growers will the primary beneficiaries of MON 863. They are expected to derive a direct yield advantage from planting hybrids containing event MON 863, as well as an indirect benefit associated with not having to apply a conventional insecticide product for control of CRW. Introduction of this technology also may lead to price reductions for conventional CRW-control insecticides. Over time some portion of these economic benefits will be passed on to consumers in the form of lower grain prices.

4.2.3 Market Share Implications

No market share implications are anticipated for seed corn producers in Australia and New Zealand.

4.2.4 Price Implications

The value of the corn yield will vary with industry demand as with other commodities. The traded price for other crop products will also remain unaffected and will fluctuate according to normal conditions.

4.2.5 Trade Implications

Australia and New Zealand are members of the World Trade Organisation (WTO) and are bound as parties to WTO agreements. In Australia, an agreement developed by the Council of Australian Governments requires States and Territories to be bound as parties to those WTO agreements to which the Commonwealth is a signatory. Under the agreement between the Governments of Australia and New Zealand on Uniform food Standards, ANZFA is required to ensure that food standards are consistent with the obligations of both countries as members of the WTO.

In certain circumstances Australia and New Zealand have an obligation to notify the WTO of changes to food standards to enable other member countries of the WTO to make comment. Notification is required in the case of any new or changed standards which may have a significant trade effect and which depart from the relevant international standard (or where no international standard exists).

Matters relating to public health and safety may be notified as a sanitary or phytosanitary notification, and other matters relating to trade may be notified as a Technical Barrier To Trade notification.

Exports

36,000 tonnes of corn were exported in 1995-96.

Imports

Due to the diverse uses of corn products there has been a requirement to import this commodity to meet the short fall. Australia imported 400 tonnes of corn products in 1995-96 as high fructose corn syrup.

Regulatory approvals in the U.S. for event MON 863 will not require segregation; thus U.S. exports of corn and corn products may contain event MON 863 without special labelling. Therefore, if event MON 863 is either not permitted or requires special labelling in Australia or New Zealand, imported corn from the U.S. will incur cost penalties. Due to these circumstances, international trade obligations must be considered. If Australia and New Zealand reject shipments of corn or corn products from the U.S., there will be consequential trade implications. The inclusion of corn and corn products containing event MON 863 in the *Food Standards Code* may be needed to satisfy international trade obligations. The WTO could regard any ban on the import of MON 863 corn and products containing MON 863 corn as a nontariff barrier to trade.

Australia and New Zealand could experience increased imports of corn and corn products from the U.S. if domestic growers are attracted to alternative crops.

Imports could also result from:

i) a requirement to secure specific corn specification for particular applications.

ii) an increased demand for HFCS by food processors; HFCS is not manufactured in Australia.

Interruptions in the supply of corn and corn products for Australian food processors, will directly affect delivery of basic foodstuffs to the Australian consumer.

4.2.6 Employment Implications

Import approval of corn event MON 863 is not expected to have any implications with regard to employment in Australia.

Study reports being submitted by Monsanto in support of this application include:

Mitchell, P. D. (2002). Yield Benefit of Corn Event MON 863. MSL-17782, an unpublished analysis conducted for Monsanto Company.

Ward, D. P. (2002). Public Interest Assessment Supporting Registration of *Bacillus thuringiensis* Cry3Bb1 Protein and the Genetic Material (Vector ZMIR13L) Necessary for its Production in Corn Event MON 863. MSL-17766, an unpublished assessment prepared by Monsanto Company.

5.0 STATUATORY DECLARATION - AUSTRALIA

I, Megan Elisabeth Shaw, 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 which might prejudice this application.

Signature _____

Declared before me..... TYLER DEMPSEY (CPA) .....

This.....25th.....day of ..... November ....., 2002

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7.0 UNPUBLISHED REPORTS BEING SUBMITTED

SD1 1) Bonnette, K. L. and P. D. Pyla (2001). An acute oral toxicity study in mice with *E. coli* produced Cry3Bb1.11098(Q349R) Protein. MSL-17382, an unpublished study conducted for Monsanto Company.

SD2 2) Cavato, T.A. and Lirette, R.P. (2001) PCR analysis and DNA sequence of the insert in corn rootworm event MON 863. MSL-17108, an unpublished study conducted by Monsanto Company.

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SD13 13) Li, M. H. and E. H. Robinson (1999). Evaluation of insect protected corn lines MON 853 and MON 859 as a feed ingredient for catfish. MSL-16164, an unpublished study conducted by Monsanto Company.

SD14 14) Mitchell, P. D. (2002). Yield Benefit of Corn Event MON 863. MSL-17782, an unpublished analysis conducted for Monsanto Company.

SD15 15) Ridley, W. R., M. A. Nemeth, J. D. Astwood, M. L. Breeze and R. Sorbet (2002). Amended report for MSL-17199: Compositional analyses of forage and grain collected from corn rootworm protected maize event MON 863 grown in 1999 U.S. field trials. MSL-17669, an unpublished study conducted by Monsanto Company.

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